Coevolution of *Heliconius* spp. and *Passiflora* spp.: A Phylogenetic Comparison.

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ABSTRACT

Although a substantial amount of research has been done on all aspects of *Heliconius* biology and their ecological interactions with *Passiflora*, there has not hitherto been a phylogenetic examination of this association for coevolution. To test the *Heliconius/Passilfora* association for coevolutionary congruence, phylogenies for each group were established and compared. The phylogeny for 14 species of Heliconiinae from Costa Rica was based on combined sequence data from rRNA ITS 2 and partial EF-1α gene regions. For the Passifloraceae, 17 host plant species were utilized to establish a phylogeny based on tRNA-Leucine and ITS 1/5.8S/ ITS 2 sequence data. The phylogenies for both groups were largely in agreement with current classification (for Passifloraceae) and previously established phylogenies. Associations with the large subgenera *Passiflora* and *Decaloba* correspond with the two major Advanced Radiation groups in *Heliconius*. Although strict congruence above subgenus level was not observed, broad scale congruence was evident. One main host shift as well as other possible explanations for lack of strict congruence are suggested.

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INTRODUCTION

COEVOLUTION AND PHYLOGENETICS

Coevolution is the ecological interactions of species that may or may not result in reciprocal genetic changes (Futuyma and Slatkin, 1983). For butterflies and plants, it was hypothesized by Ehrlich and Raven (1965) that past evolutionary interactions between intimately associated insect/plant groups may have influenced present day species correlations. Their model suggests that certain plant groups evolved antiherbivore mechanisms and this resulted in an adaptive radiation. This plant radiation was then followed by a subsequent adaptive radiation in the associated insect group that has evolved the capability to overcome those plant defenses (Ehrlich and Raven, 1965). It is through investigating these mechanisms and interactions that our understanding of coevolution can be developed. Furthermore, the examination of relationships at lower taxonomic levels (i.e. generic and species levels) may elucidate the evolutionary patterns of coevolution.

The use of molecular phylogenetics to test for topological congruence of phylogenies in suspected coevolving groups is a very recent concept attempted for only a few close associations (Clark *et al.*, 2000; Roy, 2001). Strict congruence of host and associate phylogenies would signify a continual association of species pairs seen through parallel cladogenic events (see Appendix I for Glossary of Terms). The expected result of such an association would be speciation in the host followed by speciation in the associate in a strict cospeciating process (Futuyma and Slatkin, 1983; Brooks and McLennan, 1991; Clark et al., 2000). Although this is the expected result of historically closely interacting organisms, evidence thus far indicates that complete congruence is rare, if not absent, in most associations.

The reasons for lack of coevolutionary congruence between associating lineages are as follows: Firstly, a lack of simultaneous speciations in both the host and associate groups or speciation with both ancestral and derived species remaining on the same host may occur. Secondly, following the host speciation event, extinction of the associate is likely if reciprocal adaptation does not occur. Alternatively, extinction of the host species may occur which could result in isolation and condemnation of the associate. Thirdly, host switches or host jumps are possible whereby associate lineages colonize new hosts. And finally, the duration of the association or ages of the interacting groups may not be sufficiently matched to affect each others' evolution (Mitter *et al.*, 1991).

THE CASE OF *HELICONIUS* AND *PASSIFLORA*

The *Heliconius* butterflies and their *Passiflora* host plants are consistently cited as a primary example of coevolution in the literature (Ehrlich and Raven, 1965; Benson *et al.*, 1975; Brown, 1981; Futuyma and Slatkin, 1983; Mitter and Brooks, 1983; Smiley, 1985b; Gilbert, 1971; Gilbert, 1975; Gilbert, 1991). The assessment of this association is based on the extensive coverage of the ecologically associated features of these highly intriguing and complex Neotropical communities. Equally convincing is the concordance of the presumed ages of either of the two groups. However, the strictness of this coevolutionary relationship has yet to be tested by examining the extent of congruence between their phylogenies.

The phylogenetic history of the Heliconiinae has been covered at length by several authors (Emsley, 1963; Emsley, 1965; Brown, 1981; Brower, 1994a; Brower and Egan, 1997; Penz, 1999). The most extensive of these representations is the molecular phylogeny of Brower and Egan (1997) which includes 10 genera and 58 species from several geographic

locations resulting in 54 equally parsimonious trees with a tree length of 2212 steps (CI=0.304, RI=0.556). This finding is not highly conclusive and would likely be clarified by narrowing the scope to a lesser number of species and species origin. For the Passifloraceae, no phylogeny has ever been established morphologically or otherwise for those passionvines that are hosts of the heliconiines.

REASEARCH OBJECTIVES

1. To establish a phylogeny of 14 species of Heliconiinae from Costa Rica based on ITS 2 and partial EF-1α sequence data.
2. To establish a phylogeny of 17 species of Passifloraceae based on ITS 1/5.8S/ITS 2 and tRNA-Leucine sequence data.
3. To compare the host plant and butterfly phylogenies for coevolutionary congruence.

LITERATURE REVIEW

THEORY OF COEVOLUTION

The term coevolution was popularized by Ehrlich and Raven (1964) in their fundamental paper regarding the evolutionary roles that butterflies and their host plants have played in influencing each other. Although studies in coevolution have only gained popularity within the last 30 years, the ideas behind coevolution date back to Darwin's origin of the study of evolution: "Thus I can understand how a flower and a bee might slowly become, either simultaneously or one after the other, modified and adapted in the most perfect manner to each other" (Darwin, 1859 as reviewed by Futuyma and Slatkin, 1983).

Current definitions of coevolution range from restrictive to diffuse. The restrictive interpretation, pair wise coevolution, utilized by some researchers requires the specific evolution of a characteristic in one species in response to a characteristic in another species which, in turn, has resulted in response to a characteristic in the first species. An even further restrictive limitation to this definition would be that the characteristics evolve simultaneously (Futuyma and Slatkin, 1983). In some instances, associate organisms are so intimately tied that they have cospeciated with one another as seen through phylogenetic comparison (Brooks and McLennan, 1991; Clark *et al.*, 2000). The relaxation of any of these restrictions would be a broader, diffuse coevolution, and less specific or, at the extreme, even simply evolution (Futuyma and Slatkin, 1983). An example of extreme diffuse coevolution would be the evolution of physical and or chemical defense traits against a wide spectrum of insect herbivores and the evolved capability in insects to cope and surpass those defenses in a wide array of plants.

The intimate pair wise association of *Heliconius* butterflies and their *Passiflora* host plants is heralded as an example of pair wise coevolution where each has adapted to specific traits of the other. It is this association that will be the system of study for the current thesis.

PHYLOGENETIC ASPECTS OF COEVOLUTION

The use of phylogenetic analyses and related evidence from historical associations to test for coevolution between host plants and their herbivorous insects has been a relatively recent consideration (Mitter *et al.*, 1991; Brooks and McLennan, 1991). Several characteristics are instrumental in elucidating the ecological and evolutionary processes involved in intimate insect/plant interactions. For example, the role of plant chemistry and other plant defenses and insect counter adaptations as well as the role they play in diversification of host and insect groups are important considerations for examining historical associations.

In general, there are four main issues outlined by Mitter *et al.* (1991) that are of importance in examining a potential coevolutionary relationship. Firstly, the age of the association is of importance. The older both groups are, the greater the chance that they or their ancestors have influenced each other over time. Secondly, the extent to which the insect and plant lineages have diversified in association should be represented by strict congruence of the host plant and insect phylogenies (Mitter *et al.*, 1991). Thirdly, diversification may be accelerated in plant and insect groups that have evolved mechanisms that confer defense or counterdefense. Finally, diversification in each group individually should be evidenced by advancing complexity of defenses in the plants and counter defenses in the insect (Mitter *et al.*, 1991).

Brooks and McLennan (1991) outline models and methodology by which coevolutionary host and associate groups are compared for phylogenetic congruence. The colonization model (association by colonization) involves the evolution of the plant group primarily, followed by colonization by the insect. The classical (diffuse) coevolution model (association by descent) on the other hand, is the 'arms race' ideology whereby mutually adaptive responses in the host plant and insect are what drive the coevolving ecological association (Brooks and McLennan, 1991).

LIFE HISTORY OF *HELICONIUS*

CLASSIFICATION

The Order Lepidoptera, containing the butterflies and moths, is a large and diverse Order encompassing over 11,000 species in the United States and Canada in a vast range of habitats (Borror *et al.*, 1992). The Lepidoptera are of considerable economic importance due to the phytophagous nature of their larvae. Within the Order Lepidoptera is the Family Nymphalidae (Brush-footed butterflies) which contains the subfamily Heliconiinae comprised of largely Neotropical groups. The heliconiines are differentiated from other nymphalids by their long antennae, large eyes and, notably, their narrow, elongated forewings.

For a food source, this subfamily almost exclusively utilizes host plants within the Family Passifloraceae and it is from this restricted association that this group has derived their name as the 'Passionflower butterflies' (DeVries, 1987). The distribution of the heliconiines includes the West Indies, Central and South America and the Southern United States. At

present, approximately 70 species have been identified throughout these regions and 24 of these species can be encountered in Costa Rica (DeVries, 1987).

The heliconiine larvae are the phytophagous stage and feed either alone or gregariously depending on the species (DeVries, 1987). Morphologically, the immatures show a variety of colours and patterns and the species are usually uniform in size. In addition, all heliconiine larvae are spiny with many species having irritant spines (Devries, 1987) which are thought to be a means of predator avoidance for this group.

The highly visible, brightly coloured adult heliconiines are a prominent presence in Neotropical rainforests. Morphologically, male and female heliconiines are indistinguishable except through examination of the genitalia and the location of 'stink clubs' on female individuals. These stink clubs are located adjacent to the protruding abdominal glands of the female and, in some species (such as *H. erato*) when manipulated produce an obvious pungent odour likened to phenylcarbylamine (Gilbert, 1976). This odour is present only in mated females.

Another unique feature of the heliconiines occurs in the mating behaviours of some species. Adult male heliconiines have been observed to locate and await eclosion of females from their pupae in order to gain the first copulation with the newly emerged virgin female. What accompanies this pupal mating behaviour is thought to be the transfer of the odour that the females possess from the copulating male. Gilbert (1976) hypothesized that this odour operates as an 'antiaphrodisiac' to deter subsequent matings with the female but Cornish (2001) was unable to demonstrate any such role in mated *H. erato* or *H. charithonia*.

Unlike the larvae, the adults do not consume vegetation for sustenance. Instead, the adult diet consists of nectar (utilized for flight energy and mating) and pollen, which is a rare food source that is only reported for this group (Gilbert, 1972; Murawski and Gilbert, 1986; DeVries, 1987). The plants from which the adult heliconiines obtain their pollen meals are in the family Cucurbitaceae and mainly occur in the genera *Psiguria* and *Gurania*. These vines have a lifespan of many years and are continually producing male flowers and thus are an excellent source of pollen for these butterflies with which a mutually beneficial relationship has formed (Gilbert, 1975; DeVries, 1987) including the specialized mouthparts of the adults adapted for pollen feeding (Krenn and Penz, 1998). This highly specialized pollen feeding behaviour enables these butterflies to actively accumulate pollen and extract amino acids for egg production and maintenance while functioning as pollen dispersal agents (Murawski and Gilbert, 1986; Krenn and Penz, 1998).

MÜLLERIAN MIMICRY

The wing patterning and colouration observed in *Heliconius* butterflies has often been cited as an excellent representation of Müllerian mimicry (Mallet, 1986; Mallet *et al.*, 1996). Müllerian mimicry is defined as the phenotypic close resemblance between two or more distasteful, relatively distantly related species (Müller, 1879). More specifically, Müllerian mimicry describes convergence of the different warning colouration of unpalatable sympatric species to the same pattern under selection from their predators. The end result is an umbrella of protection from predators for both models and mimics. In some *Heliconius* species, there is a geographical correlation to the observed colour pattern modifications. *H. erato* and *H. melpomene* display a great degree of parallel race formation and are therefore one of the most extensively studied pairs of species.

EVOLUTION OF *HELICONIUS*

The evolutionary history of the heliconiines has been covered extensively by Emsley (1965) and Benson *et al.* (1975). The morphological and distributional data presented by these authors show that the main radiation of *Heliconius* occurred in tropical America from primitive Nymphalidae (Benson *et al.*, 1975). The age of this group of butterflies is projected by Benson *et al.* (1975) to be over 60 million years old as estimated by historic land movements. Fossil evidence however, dates the lepidopterans as a group back to the mid-Cretaceous (approximately 100 million years ago) (Carpenter, 1954).

The primitive heliconiines are represented by six genera and 11 species and the more derived groups consist of four genera with 54 species; *Laparus* contains only one species while *Heliconius* displays the greatest diversity with 38 species (Gilbert, 1991). Based on their morphological, distributional and food plant usage data, Benson *et al.* (1975) have presumed the following evolutionary order and progression of heliconiines: in subgroup I (Primitive Genera) *Agaulis vanillae*, *Dryadula phaetusa* and *Dryas iulia* are grouped. Following this, in subgroup II (Mature Leaf Radiation) is the genus *Euides* and in subgroup III are three offshoots of *Heliconius*.

The *Heliconius* groups begin with the Early *Heliconius* Radiation in which *Laparus doris* is placed. In subgroup IV is the Advanced *Heliconius* Radiation A which contains the 'silvaniforms' (*H. hecale* and *H. ismenius*) and the 'melpomene' group, with *H. melpomene* and *H. cydno*. The final subgroup V (the Advanced Radiation B) contains *H. erato* in the 'erato' group, *H. charithonia* and *H. hortense* in the 'charithonia' group and *H. sara*, *H. sapho* and *H. eleuchia* in the 'sara-sapho' group.

SYSTEMATICS OF *HELICONIUS*

Although the taxonomy of the heliconiines has been subject to numerous revisions for more than a century, their phylogenetic relationships have only been examined over the past two decades. These phylogenetic analyses have included the combined morphological and ecological phylogeny of Brown (1981) and the molecular phylogeny of Brower and Egan (1997). Hereafter, these two phylogenetic treatments will be referred to as 'non-molecular' and 'molecular', respectively.

NON-MOLECULAR PHYLOGENY

Until the recent incorporation of molecular techniques into phylogenetic study, the most widely accepted phylogeny of the Heliconiini was that of Brown (1981). The analysis covers the 10 genera of Neotropical butterflies and includes 65 species (see Figure 1 for the phylogenetic relationships of the 14 species examined in this study according to Brown (1981)). The characters used by Brown (1981) in establishing the phylogeny include morphological features such as features of the immatures and adults, behavioural characteristics such as the use of pollen and host plants and biochemical traits including the storage of 3-hydroxykynurenine (an amino acid in the light yellow pigmentation of the wings).

The Brown (1981) phylogeny is in agreement with the classification of the Advanced Radiation B as including *H. eleuchia*, *H. sapho* and *H. sara* as most closely related (within the 'sara-sapho' group) and *H. erato*, *H. charithonia* and *H. hortense* as most closely related (within the 'erato' and 'charithonia' groups, respectively). The one exception is the placement of both *H. charithonia* and *H. hortense* together in the taxonomic grouping of the

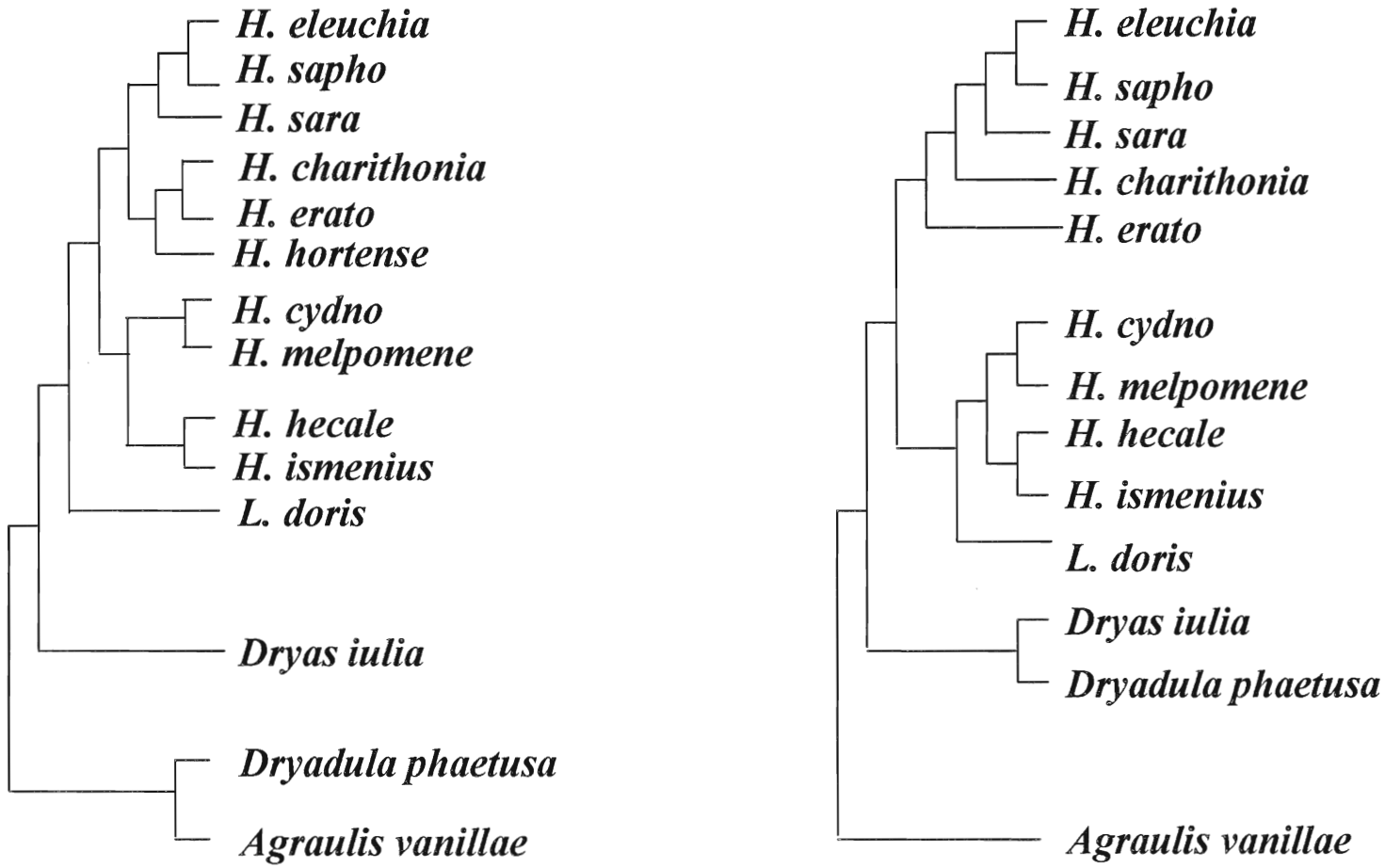

Figure 1. Phylogenies of Brown (1981) (left; based on morphological data) and Brower and Egan (1997) (right; based on molecular data) for the Heliconidae species of this study

'charithonia' group (Benson *et al.*, 1975). The sister clade to this then contains the 'melpomene-cydno' group and the 'silvaniform' group (Brown, 1981). In the taxonomical classification this clade corresponds to the Advanced Radiation A. The next ancestral taxon in both Brown (1981) and the taxonomy of heliconiines is *Laparus doris*, the Early Radiation, followed by the Basal or Primitive genera consisting of *Dryas iulia* followed by *Dryadula phaetusa* and *Agraulis vanillae* (Benson *et al.*, 1975; Brown, 1981).

Although there is almost complete concordance between the Helconiinae classification and the phylogeny of Brown (1981), the finding of Brown (1981) may not be an accurate representation of the phylogenetic relationships of the heliconiines. It is uncertain exactly how the characters used by Brown (1981), listed in the data tables, were coded for because explicit data matrices are not presented and analyses are not discussed. Therefore it is not known how the phylogenetic analysis of this complex data set was performed. In addition, on the Brown (1981) phylogeny, only a few character state changes are indicated on a small percentage of the branches of the phylogeny. Limitations aside, the Brown (1981) assessment of the Heliconiinae has been used to develop current theory on host plant-herbivore coevolution for this group (Benson *et al.*, 1975; Gilbert, 1991).

MOLECULAR PHYLOGENIES

The advent of molecular techniques has added a new dimension to the study of phylogenetics enabling the use of genetic data as characters. Lee *et al.* (1992) attempted a revision of the Brown (1981) study through the use of DNA restriction site mapping of the 18S and 28S ribosomal DNA of 11 heliconiine taxa. Also included in the analysis were 15 morphological characters obtained from the literature that were used to supplement the

molecular data. From their restriction site mapping analysis Lee *et al.* (1992) found a total of 17 restriction site characters that are listed in the data matrix for phylogenetic analysis.

Upon closer examination of their data matrix however, only 10 of the 17 molecular characters are informative and only 7 of the 15 morphological characters provide information for *Heliconius* relationships. In the phylogenetic analyses, Lee *et al.* (1992) analysed the combined data as well as analysing each data set independently to assess the relative contributions of molecular and morphological characters. The restriction site data analysis alone does not support the traditional views based on morphology whereas the morphological and combined analyses are in agreement with Brown (1981) due to the higher weighting of the character set towards the morphological data. One deviation from Brown (1981) is within the 'melpomene-cydno' complex. In the analysis of Lee *et al.* (1992), *H. melpomene* and *H. cydno* did not group together. The molecular data also fail to separate the heliconiines into pupal and non-pupal mating (see Figure 18) clades completely as is traditionally hypothesized (Benson *et al.*, 1975; Brown, 1981; Gilbert, 1983, Gilbert, 1991; Brower, 1997).

Brower and Egan (1997) present a substantial contribution to the field of Nymphalidae systematics. Brower and Egan's (1997) analysis is a revision of previous work by Brower (1994a) with the addition of taxa and DNA sequence data from a nuclear gene region. This combined phylogenetic analysis is based on 10 genera and 58 species (including 36 *Heliconius* species) from several geographic regions (see Figure 1 for the phylogenetic relationships of the 14 taxa of the current study as represented by Brower and Egan (1997)). It combines sequence data from mtDNA, from the cytochrome oxidase subunit I and II totaling 950 aligned bases and five binary gap characters, with 378 aligned characters from the protein coding gene wingless (Brower and Egan, 1997).

Phylogenetic analysis of this combined set of sequence data resulted in 54 equally parsimonious trees with a tree length of 2212 steps (CI=0.304, RI=0.556). The monophyly of *Heliconius* is supported by 12 characters, for their complete taxa set, with a branch support of four (Brower and Egan, 1997). For the most part, this topology agrees with that traditionally suggested by morphologists (Brown, 1981). One difference is the placement of *H. erato* as ancestral to *H. charithonia* and the more derived 'sara-sapho' group. One other major variation is the placement of *L. doris* as a sister taxon to the 'melpomene-cydno'and 'silvaniform' clade as opposed to the ancestral position assigned by Brown (1981) (see Figure 1). Finally, amongst the basal Heliconiinae the placement of *Dryadula phaetusa* differs between Brown (1981) and Brower and Egan (1997). However, these two phylogenies are rooted differentially with different outgroups explaining the equivocal position of this taxon.

LIFE HISTORY OF *PASSIFLORA*

CLASSIFICATION

Passionvines have long been considered to be of great economic and botanical importance. Due to their vast diversity and intricacy, *Passiflora* species have long been a 'floral marvel'. In addition, *Passiflora* produce unique aromatic fruit and reportedly produce sedatives which gives a role of both economic and medicinal importance to this intriguing plant (Killip, 1938; Moraes *et al.*, 1997). For classification, the passionvines fall under the Order Passiflorales and the Family Passifloraceae. Within the Passifloraceae there are 18 genera, four of which are located in the New World. The New World genus of primary importance is *Passiflora* which is easily the largest genus within Passifloraceae and currently contains between 455 to 465 recognized species within 24 subgenera (Killip, 1938; Vanderplank, 1996).

Specific to this study are 18 species of *Passiflora* which fall under five subgenera including the two largest subgenera *Decaloba* and *Passiflora* (see Table 1 for taxonomic classification).

GENERAL BIOLOGY

The majority of passionvines are herbaceous or woody plants that climb by way of tendrils. These tendrils are found individually in the axils of the leaves and the stems are characteristically three to five angled. One of the most distinguishing features of *Passiflora* is the vast variations observed in leaf shape throughout the genus. The leaves are always alternate though they may be undivided and transversely elliptic, orbicular, bilobed, three to five lobed, broadly ovate or narrowly linear (Vanderplank, 1996). The leaf margin is frequently entire but may be toothed in some species. In addition, the majority of *Passiflora* species possess foliar and bracteolar glands which are nectar secreting glands usually located on the petioles or along the bract margins and on the undersurface of the leaves (Killip, 1938; Vanderplank, 1996).

EVOLUTION OF *PASSIFLORA*

As passionvines have long been of interest to botanists, horticulturalists and taxonomists, their classification and evolutionary history has long been debated and is still undergoing revision as more species are continuing to be recognized (Killip, 1938; MacDougal, 1994; Vanderplank, 1996). The flowering plants or angiosperms as a whole are believed to have appeared in the Cretaceous approximately 135 million years ago (Downes and Dahlem, 1989). Fossil records date the Passifloraceae to the late Cretaceous in the Cenomanian age, approximately 96 million years ago (Chesters *et al.*, 1967; Palmer and Geissman, 1999).

Table 1. Classification of the *Passiflora* spp. examined in this study (Killip, 1938; Escobar, 1994; MacDougal, 1994; Vanderplank, 1996)

SUBGENUS V:	***DECALOBA* (*Plectostemma*)**	
Section 1:	**Cieca**	*P. coriacea*
		P. suberosa
Section 4:	**Pseudodysomia**	*P. lobata*
Section 7:	**Decaloba**	
Series 1:	***Auriculatae***	*P. auriculata*
Series 8:	***Punctatae***	*P. talamancensis*
		P. biflora
SUBGENUS XII:	***TACSONIA***	*P. mollissima*
SUBGENUS XIV:	***DISTEPHANA***	*P. vitifolia*
SUBGENUS XVII:	***PASSIFLORA* (*Granadilla*)**	
Series 1:	***Quadrangulares***	*P. quadrangularis*
		P. alata
Series 3:	***Tiliaefoliae***	*P. platyloba*
Series 5:	***Laurifoliae***	*P. ambigua*
Series 8:	***Pedatae***	*P. pedata*
Series 9:	***Incarnatae***	*P. edulis*
Series 13:	***Simplicifoliae***	*P. oerstedii*
Series 14:	***Lobatae***	*P. caerulea*
Series 15:	***Menispermifoliae***	*P. menispermifolia*
SUBGENUS XXIII:	***ASTROPHEA***	
Section 1:	**Dolichostemma**	*P. pittieri*
Section 3:	**Eustrophea**	*P. tica*

The generalized trend in angiosperm evolution is that woody plants, which are long lived and display generalized morphology and simple flowers, are more primitive. In turn, shorter lived herbaceous plants that display a more complex morphology and specialized flowers structure and are ecologically adaptable are presumed to be more recent additions in plant evolution (Benson *et al.*, 1975). In relating this evolutionary assumption to the passionvines, the subgenus *Astrophea* is the most primitive with woody vines occasionally occurring as bushes and trees. This forest canopy group is represented by large biomass of older leaves and is deemed to be very distinct by Killip (1938). The subgenus *Distephana* is considered to be the next most primitive by Benson *et al.* (1975) due to the lignified stems and shared style positioning with plants in *Astrophea*.

One of the largest and most diversified subgenera is *Passiflora* (*Granadilla* in Killip (1938)). The vines within this group are typically found along forest edges or open habitats. They are characterized by very large, well developed flowers with ornate bracts and stipules as well as extra floral nectaries in a variety of locations (Killip, 1938; Benson *et al.*, 1975; Vanderplank, 1996). The plants themselves are typically long-lived and are large and fast growing (Benson *et al.*, 1975). The majority of ornamental and fruit bearing passionvines are within this group. There are additionally several small subgenera that show variation in derived and primitive characteristics such as the vines within *Tacsonia*.

The subgenus *Decaloba* (*Plectostemma* in Killip, (1938)) is the largest of all the subgenera with over 160 species (Vanderplank, 1996). This group is considered to be the most evolved with small, herbaceous vines with photosynthetic stems and small unapparent flowers (Killip, 1938; Benson *et al.*, 1975). Although the *Decaloba* passionvines are smaller and less apparent than those in *Passiflora*, they are equally common.

SYSTEMATICS OF *PASSIFLORA*

At present, no phylogenetic analysis has been published for the Passifloraceae. All scenarios to date regarding the relationships amongst the passionvines have been based on the morphological characterization and classification of this extensive group (Killip, 1938; Vanderplank, 1996). In an examination of the genetic variation between and amongst species of *Passiflora*, Fajardo *et al.* (1998) attempted to uncover the relatedness within the genus with the use of molecular techniques. Using a total of 52 plants from 14 species (five subgenera) from several geographic locations within the Andes, Fajardo *et al.* (1998) performed a cluster analysis based on the polymorphic fragments obtained from RAPD assays.

Overall, the dendogram of genetic similarities presented correlates fairly well with the traditional classification of the five subgenera. Unfortunately, as the two largest subgenera of *Passiflora* (*Decaloba* and *Passiflora*) are only represented by two and four species respectively, this study did not include sufficient data to consider significance of the subgenus clusters formed (as noted by the authors) (Fajardo *et al.*, 1998). In addition, the subgenus *Tacsonia* occurs in the middle of *Passiflora* and the two species of *Decaloba* examined did not come out together in their analysis. In subsequent analyses, where only 12 species of three subgenera and 35 accessions were examined using cluster analysis based on RFLP data, these results were somewhat refined (Sanchez *et al.*, 1999). Unlike Fajardo *et al.* (1998), the three subgenera subsequently examined (*Decaloba*, *Tacsonia* and *Passiflora*) do cluster together. This finding more closely agrees with the traditional classification for these species. However, for a greater understanding of the interspecific relationships within this genus, a phylogenetic analysis is necessary.

THE *HELICONIUS/PASSIFLORA* ASSOCIATION AS AN EXAMPLE OF COEVOLUTION

In discussions of coevolutionary relationships, the *Heliconius/Passiflora* association is consistently cited as a primary example. These two groups are considered a clear example because the heliconiines display a high degree of host specificity and behavioural sophistication while the plants are equipped with several morphological and biochemical features that serve as defenses specific to this specialist herbivore.

DEFENSE CHARACTERISTICS OF PASSIFLORA

The passionvines possess several features, both morphological and biochemical, that are thought to have evolved due to long-term interactions with *Heliconius* (Gilbert, 1991). One of the most notable and innovative morphological traits that *Passiflora* displays is egg mimicry. These structures that mimic the eggs of *Heliconius* are produced on the stipules, tendrils, stems and meristematic tissues of several species of *Passiflora* and bear a striking resemblance to the natural, near-hatching eggs of various *Heliconius* species (Gilbert, 1975). The efficiency of this functional adaptation at reducing female oviposition was examined by Williams and Gilbert (1981). Their insectary analysis showed a strong, mainly visual response in *Heliconius* females to the presence of eggs and egg mimics and that the probability of egg lay was reduced and time to oviposition was increased (Williams and Gilbert, 1981). Therefore, this host trait is an effective post-detection defense against ovipositing *Heliconius* females.

Another structural feature in the defense repertoire of a few passionvines is the presence of hooked trichomes (Gilbert, 1971). In greenhouses containing varied species of *Passiflora* and *Heliconius*, *P. adenopoda*, unlike the other passionvines present, did not sustain any damage from heliconiine attack. In addition, when *H. erato* and *H. melpomene* larvae were

artificially placed on *P. adenopoda*, they had moved only slightly and were dead by the following day (Gilbert, 1971). Scanning electron micrographs revealed that there were hooked trichomes over the entire plant surface and that the larval prolegs had been severely snagged by several trichomes. The total damage sustained by the larvae included not only a puncture wound and cut but also loss of hemolymph (Gilbert, 1971). These hooked trichomes therefore represent an additional and highly effective feature of some *Passiflora* species against *Heliconius* larvae. However, only three species of *Passiflora* have these including *P. lobata* which interestingly, has fewer toxic chemicals than other passionvines (Smiley and Wisdom, 1985).

While the two preceding examples of *Passiflora* morphological defense traits are post-detection mechanisms, some passionvines may escape heliconiine herbivory through evading ovipositing females prior to detection. It has been argued (Gilbert, 1975) that female *Heliconius* may act as agents of visual selection on leaf shape in *Passiflora* which has resulted in the extreme variation, both intra and interspecifically, in leaf shape even within the same local habitat (Gilbert, 1975). This theory of ovipositing female heliconiines exerting visual selection on their *Passiflora* hosts, is further supported by the aforementioned example of egg mimicry development in *Passiflora*. It has also been suggested that leaf shape in some *Passiflora* species mimics the leaf shape of other prevalent tropical plants as a means of evading detection.

The heliconiines are not the only insect to form an intimate association with *Passiflora*. The presence of extrafloral nectaries on petioles, leaf surfaces, tips and margins as well as bracts in several species of passionvines has resulted in their attendance by ants and microhymenopteran parasitoids of heliconiine eggs (Benson *et al.*, 1975; Gilbert, 1975).

Extrafloral nectaries represent another morphological plant defense trait possessed by *Passiflora*. The nectar which is exuded from the nectaries is a substantial food source for a variety of ant species and in attending *Passiflora* for this nectar source, ants have been observed to kill and/or remove *Heliconius* larvae and eggs from the host plant (Benson *et al.*, 1975). In a study examining caterpillar mortality of two species of *Heliconius* (*H. ismenius* and *H. melpomene*) Smiley (1985a) found that, although overall early instar survivorship was low, ant presence resulted in an even further reduced survivorship (15% of larvae survived more than two days on plants with ants present whereas 33% of larvae survived more than two days without ants present). Therefore, the presence of extrafloral nectaries on *Passiflora* is an indirect means of defense through the attraction of ants and of *Heliconius* parasitoids.

One additional trait within the multifaceted defense regime of *Passiflora* is the use of toxic chemicals to deter potential predators. It was suggested by Ehrlich and Raven (1964) that selection by insect herbivores could result in the evolutionary diversification of plant secondary chemicals. *Passiflora* is an excellent example of such chemical diversification as these plants display a high degree of both intra- and inter-specific variation in chemical class constituents (Smiley and Wisdom, 1985). It has been suggested that this variation may be attributed to selection by heliconiines utilizing biochemical means to counter this plant defense (Gilbert, 1991). This chemical aspect of the *Passiflora/Heliconius* relationship is complex and extensive and worthy of separate examination for coevolutionary trends (Spencer, 1988; Gilbert, 1991).

In analyzing only nine species of *Passiflora* at three different life stages through phytochemical analysis, Smiley and Wisdom (1985) uncovered five chemical profiles into which these plants could be categorized according to tannin, alkaloid and cyanogenic content. Tannins in particular are generally regarded as antifeedants to most herbivores and although *H.*

melpomene (a specialist) ingested dry weight tannin concentrations of 7 to 15% in *Passiflora* leaves, there was no effect on growth rate or survival time. In contrast, the tannins from *P. alata* were ingested by the control insect *Heliothis virescens* (a generalist) proved to be very effective antifeedants at low concentrations (dosage required to reduce growth by 50% was 0.6% dry weight). Therefore, although these secondary plant chemicals may not be an effective feeding deterrent against *Heliconius*, they may serve as an effective defense against other potential herbivores.

COUNTER-DEFENSE CHARACTERISTICS OF HELICONIUS

In order to successfully continue the association with *Passiflora*, the heliconiines have developed both physiological and behavioural defense characteristics to circumvent those of *Passiflora*. The adult stage of *Heliconius* is one of the most physiologically and behaviourally sophisticated among all lepidopterans (Gilbert, 1975). One of their most advanced features that serves as an indirect means of counterdefense is the visual system. Along with their well-developed eyes, *Heliconius* displays 'traplining' behaviour whereby they visit both pollen and nectar sources with daily regularity (Gilbert, 1975).

Adult heliconiines are also routinely observed visually orienting themselves to landmarks while patrolling their home range. This characteristic is evidenced by their suitability to insectaries and enclosed conservatories. Further to this, *Heliconius* has shown 'learning' capacity through the avoidance of regions where they had previously been captured in mark and recapture experiments (Gilbert, 1991; Gilbert, 1975). In nature, female ovipositing heliconiines have been observed darting towards highly inconspicuous host-plants from several metres away (Benson *et al.*, 1975).

This specialized selective behaviour for suitable oviposition sites is another indirect defense utilized by *Heliconius* to overcome *Passiflora* defense traits such as cryptic leaf morphology and egg mimicry. Ovipositing females inspect plants extensively both visually and by tapping the plant with antennae and the use of chemoreceptors by drumming the plant surface with their forelegs. When eggs are detected, the searching and inspecting behaviour ceases as the female flies off (Williams and Gilbert, 1981). In addition to selectivity for oviposition sites, female heliconiines are also selective for the type of tissue on which they deposit their eggs. Many *Heliconius* females specifically seek meristematic tissues on or near which to oviposit.

The use of meristematic tissues by ovipositing females for the subsequently developing larvae has incurred a counterdefense capability in many ways. Firstly, the eggs and developing larvae on the plant extremities would be less apparent or accessible to patrolling predaceous ants and other proximal heliconiine larvae that would be competition for food resource or cannibalistic (Gilbert, 1975). Secondly, as the meristems themselves are often hidden within the tropical flora, the developing larvae would be less apparent to parasitoids. And finally, the young plant tissue may be less toxic, contain more nutritive material for the developing larvae and result in less biomass, time and energy required for feeding (Gilbert, 1983).

One of the most vital counterdefense mechanisms that heliconiines possess is the ability to biochemically and/or physiologically avoid the harmful effects of *Passiflora*'s array of toxic antiherbivore chemical constituents. Although the secondary chemicals of *Passiflora* proved toxic to another generalist herbivore (Smiley and Wisdom, 1985), for *H. ismenius* and *H. melpomene* there was no relationship between any of the chemical constituents of *Passiflora* host plants and growth rate or survival time. In addition, *H. melpomene*, a specialist on non-

tanniniferous *P. menispermifolia*, experienced rapid growth on *P. vitifolia*, a highly tanniniferous passionvine. As growth rate in *H. melpomene* is not enhanced on *P. menispermifolia* in comparison to others, as the digestive efficiency hypothesis would predict for a specialist, it would seem that these butterflies possess a specialized detoxification system that circumvents the toxic allelochemicals of their host plants (Smiley, 1978).

Indeed, in examining *H. sara* and its host plant *P. auriculata*, Engler *et al.* (2000) delivered the first report of the metabolism of cyanogens and therefore avoidance of toxic cyanide release. As well, in 14 other species of *Heliconius* that were all reared on their specific host plants, cyanogens were detected in individuals regardless of whether they were present in the host plant. When tested, the host cyanogens were detected in neither the butterfly larvae nor their frass. This therefore indicates that these chemicals were synthesized *de novo* in *Heliconius* (Engler *et al.*, 2000). The heliconiines have therefore efficiently overcome this barrier to herbivory with the counter strategy of surpassing the toxic effect of *Passiflora*'s cyanogenic chemical constituents.

THE ROLE OF POLLEN FEEDING

Although it is the larvae of *Heliconius* that are the host specific stage of *Passiflora*, adult heliconiines have formed their own tight association with the plants on which they pollen feed. This behaviour, which was first developed in the ancestor to *Laparus* and *Heliconius*, is unique amongst all lepidopterans (Gilbert, 1972). Pollen feeding occurs in the one species of *Laparus* and in the 38 species of *Heliconius* and has resulted in a dramatic increase in free amino acids utilizable by the adult stage (Gilbert, 1991). The end result of this innovation has

been a transfer of the accumulation of reproductive resources from the larval to the adult stage. As the larvae are particularly vulnerable to invertebrate and bird predators this shift could result in reduction in the time of exposure through reduced foraging requirements by the larvae (Gilbert, 1972; Gilbert, 1991).

The cyanogenic nature of heliconiines is the key factor in their distastefulness to their predators, which is also correlated with pollen feeding (Gilbert, 1972; Gilbert, 1991). It has been suggested that the cyanogens that heliconiines possess are manufactured from the amino acid precursors which are obtained from pollen feeding (Gilbert, 1991). It would therefore be this use of pollen which promoted their unpalatable nature, aposematism and mimicry as well as the capability of utilizing *Passiflora* as a food source. With the development of these capabilities, the possibility for further physiological and behavioural specialization would be enhanced (Gilbert, 1991).

PROBABLE COURSE OF COEVOLUTION

As the ancestral *Passiflora* would have been exposed to a diverse array of herbivores, one of the primary stages in their evolution would likely have been the development of defensive chemicals. As these chemical constituents increased in variety, those herbivores capable of overcoming the array of plant chemical defenses would decrease. In order to further defend against specialist herbivores such as protoheliconiines, selection would then favour the development of morphological and mechanical defense mechanisms (Gilbert, 1983). The next step in this hypothesized coevolutionary arms race is suspected to have occurred in the ancestral heliconiines in order to overcome these newly acquired plant defenses.

It is suggested by Brown (1981) that the genus *Heliconius* evolved relatively recently from primitive heliconiine stock and subsequently endured dramatic adaptive radiation. There are two key innovations that have been implicated in this radiation: firstly, the increased reproductive lifespan and resultant investment by adult *Heliconius* to the reproductive effort that was achieved by pollen feeding (Gilbert, 1972; Gilbert, 1991); and secondly, the use of the young meristematic tissues of *Passiflora* (Benson *et al.*, 1975). The evolution of these innovations then would have enabled selection for further behavioural sophistication in *Heliconius* such as discriminatory oviposition.

The development of highly conspicuous yellow eggs to deter other ovipositing females may then have enabled the next coevolutionary step of egg mimicry in *Passiflora* (Williams and Gilbert, 1981). This innovation in *Passiflora* is thought to be a relatively recent elaboration as only approximately 10 species possess egg mimics and the occurrence of the egg mimicry trait is geographically variable. In addition, occurrence of egg mimics in different subgenera and on different structures such as buds, stipules and nectaries implies several independent origins for this feature (Gilbert, 1983). Thus far, the coevolutionary responses of *Heliconius* to egg mimicry in *Passiflora* are yet to be examined.

MATERIAL AND METHODS

SAMPLE ORIGIN AND DNA EXTRACTION - HELICONIINAE

The 14 Heliconiinae species examined in this study are listed in Table 2. These samples were obtained from the Niagara Parks Commission Butterfly Conservatory. All specimens were received from Costa Rica (captive bred stock) unless otherwise stated (see Table 2 for country of origin). An attempt was made to use only Costa Rican samples in order to isolate the host plant data to one geographic region as outlined in DeVries (1987). One individual of each species was utilized except where discrepancies in sequencing results occurred (see RESULTS: SEQUENCE ALIGNMENT AND CHARACTER SELECTION – *HELICONIUS* section).

All specimens were identified to species (according to the descriptions of DeVries, 1987) and prepared for live dissection. Specimens were immobilized by freezing for approximately one minute at which time wings were removed and the body was pinned through the thorax and posterior abdominal region in a Petri dish lined with Sylguard® 184 Silicone Elastomer. The specimen was then submerged in BDH Chemicals Ltd. Ringer's solution (prepared according to manufacturer's instructions) and an anterioposteriad incision was made to expose the abdominal tissues. Approximately 25 milligrams of fat body was then extracted and placed in a labeled 1.5 ml Eppendorf tubule.

Genomic DNA was extracted by grinding the insect fat body in Lysis buffer (Buffer ATL in the Qiagen DNeasy™ Tissue Kit) with an Eppendorf pestle. The complete DNA extraction was performed using the Qiagen DNeasy™ Tissue Kit according to manufacturer's instructions (DNeasy™ Tissue Kit Handbook; April 1999).

Table 2. List of *Heliconius* spp. used for phylogenetic analysis and their respective host plant *Passiflora* spp. listed by source. DeVries (1987) = Costa Rica only. Vanderplank (1996) and Benson *et al.* (1975) = worldwide host plants for each heliconiine.

Heliconiinae spp.	(DeVries, 1987)	(Vanderplank, 1996)	(Benson *et al.*, 1975)
Agraulis vanillae	***P. foetida*** ***P. quadrangularis*** ***P. ligularis*** ***P. costaricensis*** ***P. auriculata***	***P. foetida*** ***P. morifolia*** ***P. ligularis*** ***P. auriculata*** ***P. menispermifolia*** ***P. quadrangularis*** ***P. costaricensis***	***P. foetida*** ***P. vitifolia*** ***P. edulis*** ***P. costaricensis***
Dryadula phaetusa	***P. talamancensis***	***P. morifolia*** ***P. talamancensis***	***P. vitifolia***
Dryas iulia	***Decaloba (Plectostemma)*** ***P. vitifolia*** ***P. platyloba***	***P. talamancensis*** ***P. biflora*** ***P. morifolia*** ***P. punctata*** ***P. organensis*** ***P. trifasciata*** ***P. platyloba*** ***P. vitifolia*** ***P. caerulea*** **Many other** ***Passiflora* spp.**	***P. coriacea*** ***P. biflora*** ***P. costaricensis***
L. doris	***P. ambigua***	***P. ambigua***	***P. ambigua***
H. charithonia (Brower, 1994b)	***P. lobata***	***P. colimensis*** ***P. adenopoda*** ***P. morifolia*** ***P. lobata***	***P. adenopoda*** ***P. lobata***
H. melpomene	***P. oerstedii*** ***P. menispermifolia***	***P. oerstedii*** ***P. menispermifolia***	***P. menispermifolia***
H. cydno	***P. vitifolia*** ***P. biflora*** **Most other** ***Passiflora* spp.**	***P. vitifolia*** ***P. biflora*** ***P. caerulea*** **Many other** ***Passiflora* spp.**	***P. vitifolia*** ***P. quadrangularis*** ***P. ambigua*** ***P. oerstedii*** ***P. coriacea*** ***P. auriculata*** ***P. costaricensis***
H. erato	***P. talamancensis*** ***P. coriacea*** ***P. biflora***	***P. morifolia*** ***P. coriacea*** ***P. biflora*** ***P. talamancensis***	***P. coriacea*** ***P. auriculata*** ***P. biflora***
H. hecale	***P. oerstedii*** ***P. vitifolia*** ***P. auriculata*** ***P. platyloba***	***P. oerstedii*** ***P. vitifolia*** ***P. auriculata*** ***P. platyloba***	***P. vitifolia*** ***P. platyloba***
****H. ismenius*** *only El Salvador available	***P. alata*** ***P. pedata*** ***P. ambigua*** ***P. platyloba***	***P. alata*** ***P. ambigua*** ***P. platyloba*** ***P. pedata***	***P. serratifolia***
H. sara	***P. auriculata***	***P. auriculata***	***P. auriculata***
H. sapho	***P. pittieri***	***P. pittieri***	**Unknown**
H. eleuchia	***P. tica***	***P. tica***	**Unknown**
H. hortense (El Salvador)	**N/A**	**N/A**	***P. trinifolia***

SAMPLE ORIGIN AND DNA EXTRACTION – PASSIFLORACEAE

The 18 *Passiflora* species utilized in this study are listed in Table 3 along with the source of the specimen and country of origin where known. Genomic and chloroplast DNA were extracted together by grinding a small amount of leaf tissue (approximately 80 mg of wet-weight starting material where fresh samples were used and, approximately 20 mg of dry leaf material where dried herbarium samples were used) into a fine powder with a mortar and pestle with two applications of liquid nitrogen. The powder was transferred to a 1.5 ml Eppendorf tubule and the remainder of the DNA extraction was performed using the Qiagen DNeasy™ Plant Mini Kit according to the manufacturer's instructions (DNeasy™ Plant Mini Kit Handbook; August 2000).

The following steps were included in the protocol:

(1) the AP1 buffer was heated to 65°C to prevent precipitates from forming.

(2) the amount of AP1 and AP2 buffers utilized were doubled to avoid the formation of a viscous lysate which would inhibit optimal lysis.

(3) following the ice incubation in Step 4, the lysate was centrifuged for five minutes at full speed to remove the precipitates which could have sheared the DNA during the filtration of the lysate.

(4) after the two wash steps were performed, one additional wash with 500µl of 96-100% ethanol was carried out to remove any dark green or yellow colour from the DNeasy column membrane that may have been transferred to the eluate.

(5) following this final wash the DNeasy column was centrifuged for two minutes at maximum speed to ensure a dry membrane.

(6) the volume of Buffer AE utilized was increased to 200µl and incubated at room temperature for five minutes prior to centrifugation to increase the yield of DNA.

PCR AMPLIFICATION AND DNA SEQUENCING – HELICONIINAE

Two gene regions were isolated from the genomic DNA isolated from 14 species of Heliconiinae. Firstly, the 5.8S rRNA gene and the flanking ITS 1 and ITS 2 regions were amplified using the primers of Brockhouse *et al.* (1993) (see Appendix A for PCR reaction specifics; see Appendix B for all PCR protocols; see Table 4 for all primer sequences and melting temperatures (Tm)). Secondly, the second half of the EF-1 alpha gene was amplified using the primers efM51.9 and efrcM4 of Monteiro and Pierce (2001). These two gene regions were selected due to their proven utility in several phylogenetic studies of other Lepidoptera. Within another Nymphalidae group, *Bicyclus*, EF-1α proved useful for tree-tip resolution despite the lower rate of evolution in this protein coding gene (Monteiro and Pierce, 2001). The EF-1α gene has proven effective at resolving relationships at the species level in *Papilio* (Vane-Wright *et al.*, 1999). The ITS 1 gene region was also used by Vane-Wright et al. (1999) in combination with EF-1α where well resolved trees were derived.

Amplifications were performed using an MJ Research PTC-200 Peltier Thermal Cycler DNA Engine. Following amplification, all PCR products were analyzed by gel electrophoresis on a 1% agarose gel. Once the PCR products were visualized as individual concise bands they were purified using the QIAquick® PCR Purification Kit (see QIAquick® Spin Handbook (April 2000) for the protocol followed).

Table 3. Sample origin, source and code information for the Passifloraceae species examined.

Passifloraceae Species	Sample Source	Sample Origin	Sample Code
P. alata	John Vanderplank, UK	Leiden Botanical Gardens, Holland	1031
P. ambigua	NPC Butterfly Conservatory	Unknown	N/A
P. auriculata	John Vanderplank, UK	Wild, French Guyana	1357
P. biflora	NPC Butterfly Conservatory	Wild, Costa Rica	N/A
*P. caerulea**	P.S.I., Netherlands	Unknown	N/A
P. coriacea	John Vanderplank, UK	Cultivated, UK and Europe	1037
*P. edulis**	P.S.I., Netherlands	Unknown	N/A
P. lobata	John Vanderplank, UK	Wild, Ecuador	1347
P. menispermifolia	John Vanderplank, UK	Unknown	1151
P. mollissima	Elizabeth Ossowski, UK	Unknown	N/A
P. oerstedii	John Vanderplank, UK	Wild, Venezuela	1166
P. pittieri	John Vanderplank, UK	Wild, Costa Rica	1172
P. platyloba	John Vanderplank, UK	Horticultural, USA	1177
P. quadrangularis	John Vanderplank, UK	Wild, French Guyana	1368
P. suberosa	NPC Butterfly Conservatory	Wild, Florida, USA	N/A
P. talamancensis	John Vanderplank, UK	Unknown	1286
P. tica	Missouri Botanical Gardens	San Jose, Costa Rica	Morales 5267
P. vitifolia	NPC Butterfly Conservatory	Unknown	N/A

* Denotes samples grown from seed

Table 4. Primers used for PCR and/or sequencing in Heliconiinae species.

Primer Name	Source	Sequence (5' - 3')	Total Length	Tm
¤B-ITS 1	Brockhouse *et al.* (1993)	GTTGGTTTCTTTTCCTC	17	48.0°C
B-ITS 2	Brockhouse *et a*l. (1993)	TCGTAACAAGGTTTCCG	17	50.0°C
*ITS2-INT	Ossowski and Hunter (unpubl.)	CTGCGCGTCATAGTGTGAAC	20	64.5°C
efM51.9	Monteiro and Pierce (2001)	CARGACGTATACAAAATCGG	20	57.9°C
efrcM4	Monteiro and Pierce (2001)	ACAGCVACKGTYTGYCTCATRTC	23	59.0°C

¤ Primer name modified for this study with prefix 'B-'

* Used only for sequencing of PCR product

Table 5. Primers used for PCR and/or sequencing in Passifloraceae species.

Primer Name	Source	Sequence (5' - 3')	Total Length	Tm
¤W-ITS 1	White *et al.* (1990)	TCCGTAGGTGAACCTGCGG	19	62.0°C
*W-ITS 2	White *et al.* (1990)	GCTGCGTTCTTCATCGATGC	20	68.1°C
*W-ITS 3	White *et al.* (1990)	GCATCGATGAAGAACGCAGC	20	68.1°C
W-ITS 4	White *et al.* (1990)	TCCTCCGCTTATTGATATGC	20	58.0°C
cB49317	Taberlet *et al.* (1991)	CGAAATCGGTAGACGCTACG	20	64.4°C
dA49855	Taberlet *et al.* (1991)	GGGGATAGAGGGACTTGAAC	20	61.3°C

¤ Primer name modified for this study with prefix 'W-'

* Used only for sequencing of PCR product

The sequencing of the PCR products was performed at York University Core Molecular Biology and DNA Sequencing Facility in Toronto, Ontario with the primers that were utilized in the PCR reactions. As the combination of the 5.8S rRNA gene and the flanking ITS 1 and ITS 2 regions was approximately 1800 base pairs (bp), sequencing with the B-ITS 1 and B-ITS 2 primers of Brockhouse *et al.* (1993) alone was not successful in achieving a double stranded overlapping sequence. Therefore, an internal primer was designed (Ossowski and Hunter (unpubl.) in Table 4) from a region of similarity of the longest B-ITS 1 sequences obtained. This internal primer ITS2-INT was used for sequencing the ITS 2 region from all 14 species of Heliconiinae. See Figure 2 for a diagrammatic representation of primers positions, directions and regions of the ITS 1 / 5.8S / ITS 2 region.

PCR AMPLIFICATION AND DNA SEQUENCING – PASSIFLORACEAE

Two gene regions were isolated from 18 species of Passifloraceae in this study. The tRNA-Leu intron was isolated from chloroplast DNA using the cB49317 and dA49855 primers of Taberlet *et al.* (1991) (see Appendix A for PCR reaction specifics; see Appendix B for all PCR protocols; see Table 5 for all primer sequences and melting temperatures (Tm)). The second region examined was the 5.8S rRNA gene and the flanking ITS 1 and ITS 2 regions which was amplified using the ITS 1 and ITS 4 primers of White *et al.* (1990) called here W-ITS 1 and W-ITS 4. In *Passiflora*, the tRNA-Leu intron and the ITS 1 / 5.8S / ITS 2 gene regions were selected as they have both been successfully utilized in other plant molecular phylogenetic studies (Bailey and Doyle, 1999; Douzery *et al.*, 1999; Taberlet *et al.*, 1991).

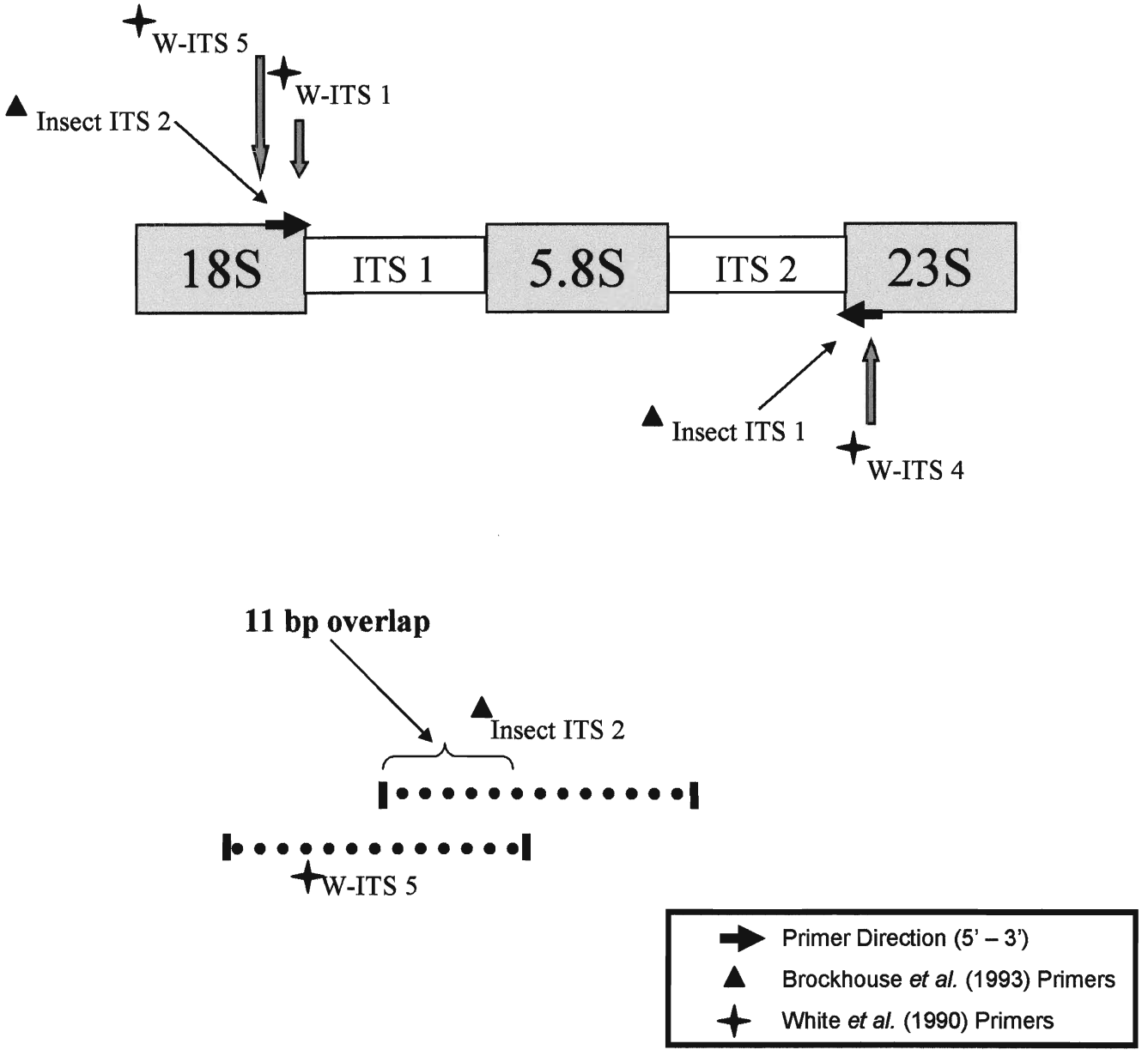

Figure 2

Diagrammatic representation of the ribosomal DNA 5.8S subunit positioning, the flanking ITS 1 and ITS 2 regions, and the primer locations for primers used in this study.

All amplification, visualization and sequencing of PCR products was performed as described above for Heliconiinae. As the sequencing results of the 5.8S rRNA gene and the flanking ITS 1 and ITS 2 regions were inadequate with only W-ITS 1 and W-ITS 4 primers, the primers ITS 2 and ITS 3 of White *et al.* (1990) (henceforth called W-ITS 2 and W-ITS 3) were included to improve sequencing results. This addition resulted in four sequences from which to determine the complete double-stranded sequence of this region. See Figure 2 for a diagrammatic representation of primer positions, directions and location of the ITS 1 / 5.8S / ITS 2 region.

SEQUENCE ANALYSIS AND ALIGNMENT

All of the text sequences obtained from the sequencing analysis were converted into BioEdit© Sequence Alignment Editor version 5.0.9 (Hall, 1999) for further manipulation. Each pair of sequences obtained for each species (and each gene region) was examined individually. Firstly, for each species the forward and reverse sequences, from the respective primers, were converted into BioEdit© format and the reverse complements were made of the reverse primer sequences. Each set of sequences was then aligned and examined for consistency. Any differences observed between the two sequences were examined further using the respective chromatograms and, where no conclusive results were possible 'N's were entered into the final sequence. This final sequence was then included in the interspecies alignment for each gene region.

All four alignments for each of the four gene regions were performed by eye. In the two Heliconiinae sequence sets, *Agraulis vanillae* was utilized as the outgroup and in the two Passifloraceae sequence sets, *Sphaerocardamum nesliiforme* sequences were obtained from

the GenBank (Accession numbers AF055263 for the tRNA-Leucine intron and AF055195 for the ITS 1 / 5.8S / ITS 2 gene region) and utilized as outgroup sequences. This species was selected as the tRNA-Leu intron and ITS 1/5.8S/ITS 2 regions have already been sequenced (Bailey and Doyle, 1999). Also, this species is outside the family Passifloraceae yet is classified under the same subclass Rosidae as Passifloraceae. From these alignments, regions of definitive alignment were selected (see Appendix C, D, E and F for each alignment and Tables 6, 7, 8 and 9 for the alignment region details). For the partial EF-1α gene region, the amino acid sequences were also established in BioEdit© to examine nucleotide changes by position and any resulting amino acid changes (see Table 10) for weighting in the phylogenetic analysis (see below).

PHYLOGENETIC ANALYSIS USING PARSIMONY

Parsimony methods of directly estimating phylogenies from character data have been the most widely used by far (Swofford and Olsen, 1990). The maximum parsimony method utlizes the principle of minimizing the number of evolutionary changes required to explain the data. In the phylogenetic analysis, the optimal tree is therefore the tree that requires the fewest number of character state changes. The maximum parsimony criterion was therefore employed in this study as the primary phylogenetic analysis of the sequence data.

Following the alignment of all sequences for each of the four gene regions, the four data sets were saved in Phylip 4 format to be converted into a MacClade version 3 (Maddison and Maddison, 1992) data matrix. Within MacClade, all data were set to standard DNA (IUPAC) notation. For the EF-1α data set, the data matrix was set as a coding region and the coding positions were calculated. In regions where the outgroup sequence was not in

agreement with the ingroup alignment, the outgroup sequence was removed (see Tables 6, 7, 8 and 9). Following this selection of regions to be included for phylogenetic analysis, the MacClade option of excluding uninformative characters was selected. To provide strength to the outgroup, characters within the alignment regions were then selected that supported this placement. All other characters were weighted equally with the exception of the EF-1α data

Table 6. Region of Insect ITS 2 alignment used in phylogenetic analysis

Alignment Region	Total Number of Characters
250	1 (1)
256	1 (1)
300 to 320	4
*321 to 332	8
373 to 489	16 (1)
491 to 498	3
532 to 579	8
**580 to 703	15
704 to 790	12 (2)
TOTAL	**68**

* Denotes regions with *A. vanillae*, *Dryadula phaetusa* and *Dryas iulia* sequences removed

** Denotes regions with *A. vanillae*, *Dryadula phaetusa, Dryas iulia* and *H. erato* sequences removed

() Number in parentheses represent number of characters selected to give strength to the outgroup

Table 7. Region of EF-1α alignment used in phylogenetic analysis

Alignment Region	Total Number of Characters
38 to 478	46 (8)

() Number in parentheses represent number of characters selected to give strength to the outgroup

Table 8. Regions of WITS alignment used in phylogenetic analysis

Alignment Region	Total Number of Characters
45 to 72	7 (3)
73 to 140	6 (6)
221 to 250	9 (3)
298 to 307	3 (1)
339 to 340	2 (2)
353 to 530	12 (3)
537 to 547	6 (1)
557	1
581 to 676	24 (11)
*677 to 778	29
TOTAL	**99**

* Denotes regions with the outgroup sequence removed
() Number in parentheses represent number of characters selected to give strength to the outgroup

Table 9. Region of tRNA-Leucine alignment used in phylogenetic analysis

Alignment Region	Total Number of Characters
20 to 118	7 (4)
154 to 173	8 (7)
∞175 to 181	1
185 to 273	14 (9)
292 to 315	5 (3)
*323 to 437	12
463 to 529	16 (10)
∞531 to 533	1
*534 to 560	3
561 to 650	7 (4)
TOTAL	**74**

∞ Denotes regions coding gaps
* Denotes regions with the outgroup sequence removed
() Number in parentheses represent number of characters selected to give strength to the outgroup

Table 10. Number of informative changes occurring at each of the three positions for the partial EF-1α gene sequences

	1st Position	2nd Position	3rd Position
Total number of changes	2	1	35
Number of changes resulting in an amino acid changes	0	1 (Lys to Arg)	1 (Glu to Asp)

set where nucleotide changes resulting in an amino acid change where weighted more heavily. To confirm that each of the four data sets was significantly more ordered than random data would be, the skew in the distribution of tree lengths for each gene region (g1 statistic) was measured. This was performed using PAUP 4.0 beta 10 (Swofford, 1993; 2002) GenerateTrees option with 1000 trees generated. Using the critical value tables for four-state character data as listed in Hillis and Huelsenbeck (1992) the significance of the g1 statistics obtained was examined.

All data sets were first analyzed individually and then by combining the Heliconiinae ITS 2 and partial EF-1α data as well as the Passifloraceae tRNA-Leu intron data and the ITS 1/5.8S/ITS 2 data for a 'total evidence' analysis (Huelsenbeck *et al.*, 1996). Prior to combining the two butterfly data sets (or two plant data sets), the partition homogeneity test of PAUP 4.0 beta 10 (Swofford, 1993; 2002) was utilized to determine whether the two data sets for each were the same. The null hypothesis of congruence is tested in partition homogeneity analyses by comparing a set of random partitions from the character matrices to each other a specified number of times (Farris *et al.*, 1994). In each of the two homogeneity tests, 100 replicates were performed. As the two host plant and two insect data sets were not significantly different, the data sets were combined to form one Heliconiinae and one Passifloraceae data set for phylogenetic analysis.

The MacClade matrices were then transformed into PAUP 4.0 beta 10 where the data were analyzed to establish the most parsimonious phylogenies achieved from these data. Within PAUP, the outgroups were redefined (as above) and heuristic searches were performed with tree bisection-reconnection swapping (TBR). Also, the initial data were unweighted and unordered (with the exception of the EF-1α data set). ACCTRAN

optimization was used for all Maximum parsimony trees shown. When multiple equally parsimonious trees (MEPT) were derived, the data were reweighted based on the rescaled consistency index and a heuristic search was again performed. The data were continually reweighted until the resulting number of equally parsimonious trees (EPT) was no longer lowered. All trees were saved and length and fit measures were recorded. When final data analyses resulted in MEPT, strict, semi-strict and 50% majority rule consensus trees were examined and the original tree that corresponded to one of the consensus trees was selected. For all final trees, 1000 bootstrap replicates were performed to obtain bootstrap values for tree nodes. In addition, Bremer Decay Indices (BDI) were calculated to establish nodal support (Bremer, 1988). The BDI values represent the number of treelength step increases required to collapse each node in the consensus tree. All final trees were recreated in MacClade and manipulated to match those produced in PAUP.

PHYLOGENETIC ANALYSIS USING MAXIMUM LIKELIHOOD

After the parsimony analysis was performed, a maximum likelihood analysis was done as a supplementary analysis for comparison with the parsimony analysis results. The maximum likelihood method of inferring phylogenies utilizes a concrete model of the evolutionary process (Swofford and Olsen, 1990). The selected model specifies the probabilities of character-state changes over evolutionary time. The optimal tree derived from maximum likelihood analysis is therefore the one that maximizes the statistical likelihood that the selected evolutionary model produced the observed character-state data. For the maximum likelihood analysis of the current study, the Hasegawa-Kashino-Yano (1985) (HKY) model of nucleotide substitution was used with variable base frequencies and

variable transition and transversion frequencies. In PAUP, a heuristic search was performed and the Best Tree Score or ln likelihood value was obtained (ln(L)). For the partial EF-1α data set, the previously applied weighting scheme was ignored. For the tRNA-Leucine data set, the gap characters were removed as only DNA data sets are allowed in maximum likelihood analyses. Using the Describe Trees command in PAUP, the Transition/Transversion ratio (Ti/Tv) was also obtained. Following the derivation of the maximum likelihood trees for the four data sets, a comparison was done with the results achieved from the parsimony analysis.

PHYLOGENETIC COMPARISON FOR CONGRUENCE – *HELICONIUS/PASSIFLORA*

Following the establishment of the two final phylogenies for *Heliconius* and *Passiflora*, the comparison was performed to test for coevolutionary congruence. Two methods were employed to test for congruence. Firstly, a simple comparison of topology was used to identify matching regions. Secondly, a host cladogram analysis was performed (Brooks and McLennan, 1991); this method is an ecological reconstruction of the phylogenetic relationships of the host plant and its closely associated herbivorous insect. After the Heliconiinae phylogeny was established the phylogenetic relationships were numbered (i.e. each taxon and each internal branch of the tree was assigned a number). Therefore, each heliconiine was assigned a binary code indicating its identity and common ancestry. These binary codes were inserted into a data matrix beside the *Passiflora* host plants on which they feed. Using this list of host plants with their respective heliconiine binary code, a new 'host cladogram' was established using MacClade and PAUP 4.0 beta 8a to create the data matrix and perform the phylogenetic analysis. This creates a picture of the

historical involvement of *Passiflora* in the evolution of these Heliconiinae. Finally, the host cladogram was compared with the *Passiflora* phylogeny based on tRNA-Leu intron data and the ITS 1/5.8S/ITS 2 data for congruence.

RESULTS

PCR AMPLIFICATION – *HELICONIUS*

Using the primers of Brockhouse *et al.* (1993) the resultant PCR fragment amplified was 1800 base pairs in length as visualized on agarose gels (see Figure 3). As sequencing of this entire region failed, an internal primer, ITS2-INT (Ossowski and Hunter (unpubl.)), was utilized to sequence the ITS 2 region only. The length of the ITS 2 region sequenced showed some length polymorphisms among the 14 species of heliconiines (see Table 11). The partial EF-1α gene region amplified with the primers of Monteiro and Pierce (2001) isolated a PCR fragment of approximately 550 base pairs in length as shown in Figure 4. The sequence obtained from primers efM51.9 and efrcM4 were only slightly variable in length (see Table 11) for the 14 species of heliconiines.

PCR AMPLIFICATION – *PASSIFLORA*

For the 17 species of *Passiflora* examined in this study the primers of White *et al.* (1990) were implemented in PCR to amplify the complete ITS 1/5.8S/ITS 2 gene region. Primers W-ITS 1 and W-ITS 4 amplified a fragment of approximately 700 base pairs in length as seen in Figure 5. For sequencing of this region two additional primers were included, W-ITS 2 and W-ITS 3, and all four sequences from all 17 species showed length variation (see Table 12). Similarly, the cB49317 and dA49855 primers of Taberlet *et al.* (1991) isolated a gene region of approximately 650 base pairs from the chloroplast DNA of the 17 species of *Passiflora* (see Figure 6). The different species showed only slight variation in sequence length for each primer sequence as can be seen in Table 12.

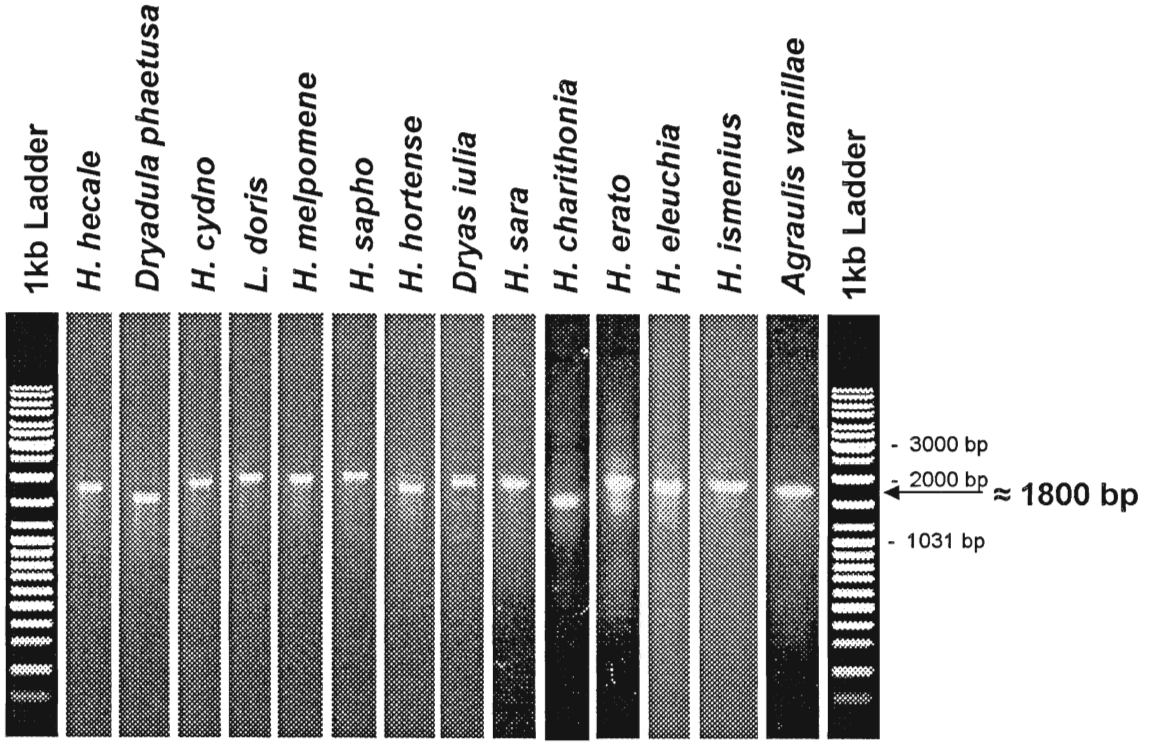

Figure 3. ITS1/5.8S/ITS2 PCR products from 14 species of Heliconiines visualized on a 1% agarose gel stained with ethidium bromide. Far right and left lanes show the molecular weight marker (GeneRuler™ 100 – 10000 bp DNA Ladder) with base pair sizes indicated on the right of the figure.

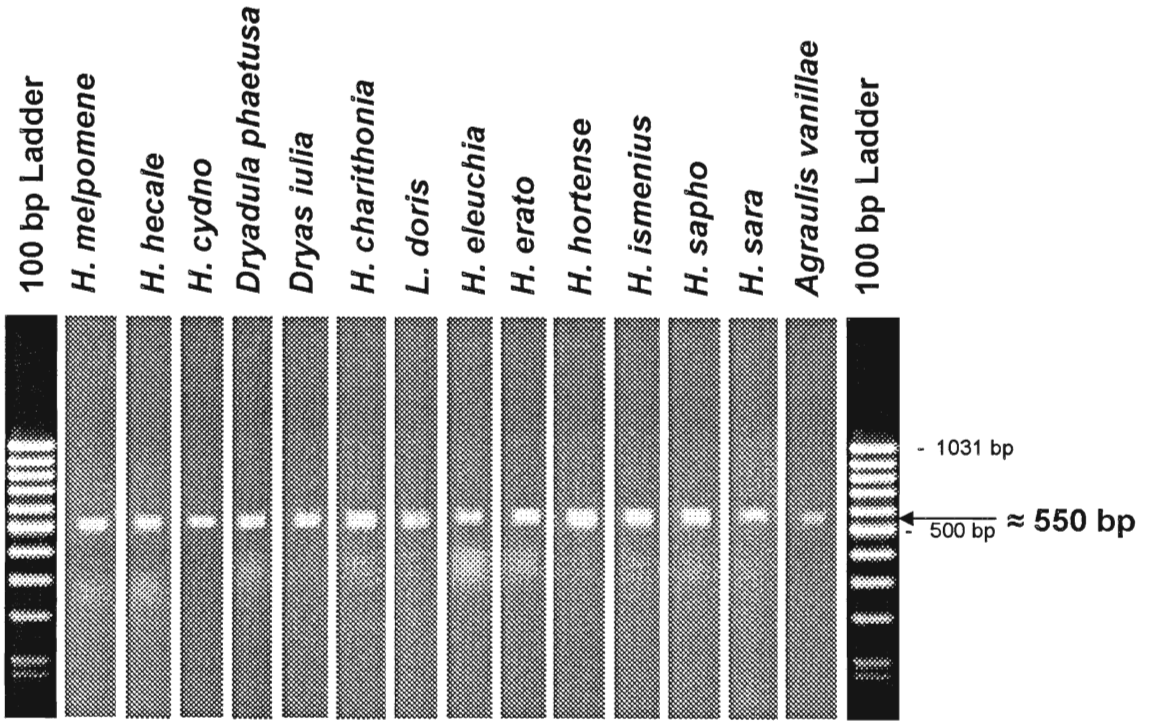

Figure 4. Partial EF-1α PCR products from 14 species of Heliconiines visualized on a 1% agarose gel stained with ethidium bromide. Far right and left lanes show the molecular weight marker (GeneRuler™ 100bp DNA Ladder) with base pair sizes indicated on the right of the figure.

Table 11. Lengths of sequences obtained from each of the primers for the two gene region examined in the 14 species of Heliconiinae. (All species are from Costa Rica unless otherwise indicated)

Heliconiinae Species	**B-ITS1**	**ITS2-INT**	**B-ITS 2** dsDNA	**efM51.9**	**efrcM4**	**Partial EF-1α** dsDNA
Agraulis vanillae	710	701	701	534	531	509
Dryadula phaetusa	705	650	672	546	544	513
Dryas iulia	403	497	500	534	479	509
Heliconius charithonia	670	706	704	560	540	532
H. cydno	744	749	741	537	531	529
L. doris	622	620	491	523	547	500
H. eleuchia	639	634	442	520	533	496
H. erato	352	562	562	532	547	511
H. hecale	720	718	692	537	530	505
H. hortense (El Salvador)	670	718	599	535	532	512
H. ismenius(El Salvador)	566	517	406	532	533	599
H. melpomene	790	714	698	529	530	504
H. sapho	622	682	518	501	480	441
H. sara	550	704	704	527	519	512

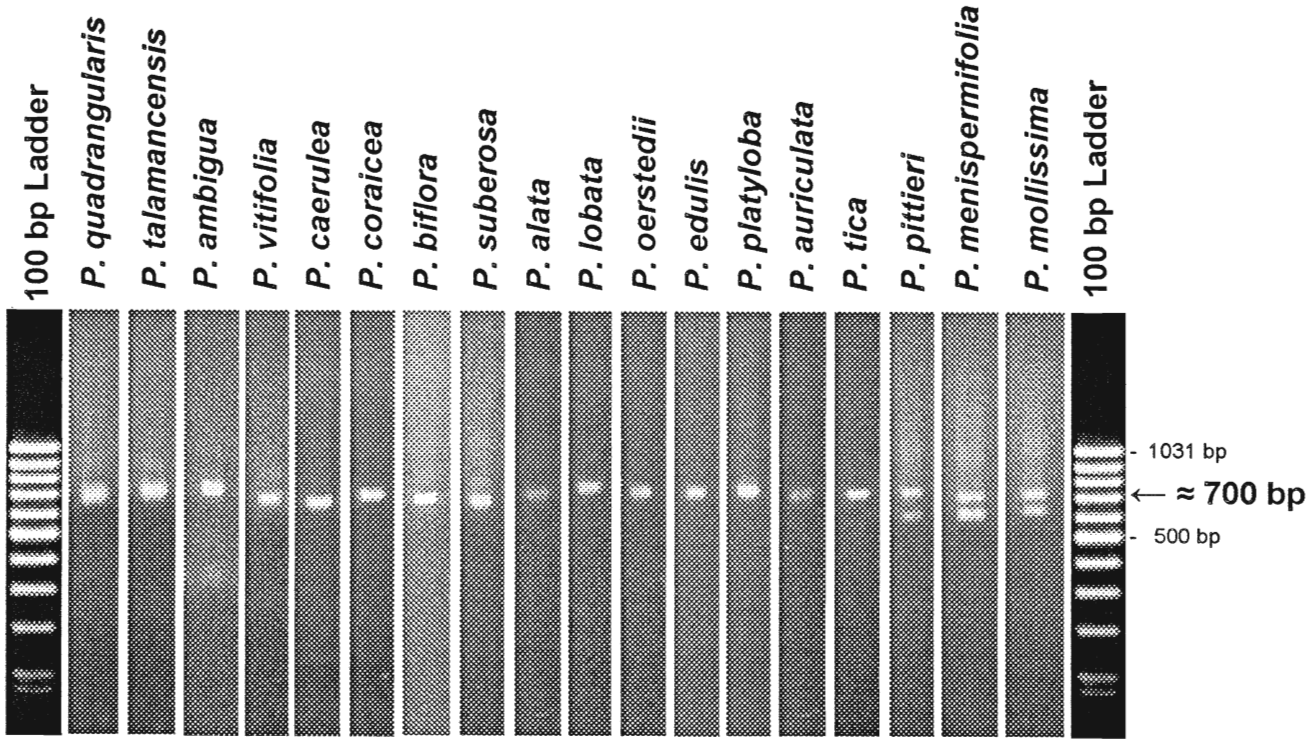

Figure 5. ITS1/5.8S/ITS2 PCR products from 18 species of *Passiflora* visualized on a 1% agarose gel stained with ethidium bromide. Far right and left lanes show the molecular weight marker (GeneRuler™ 100bp DNA Ladder) with base pair sizes indicated on the right of the figure. Number in bold with arrow indicates approximate size of bands.

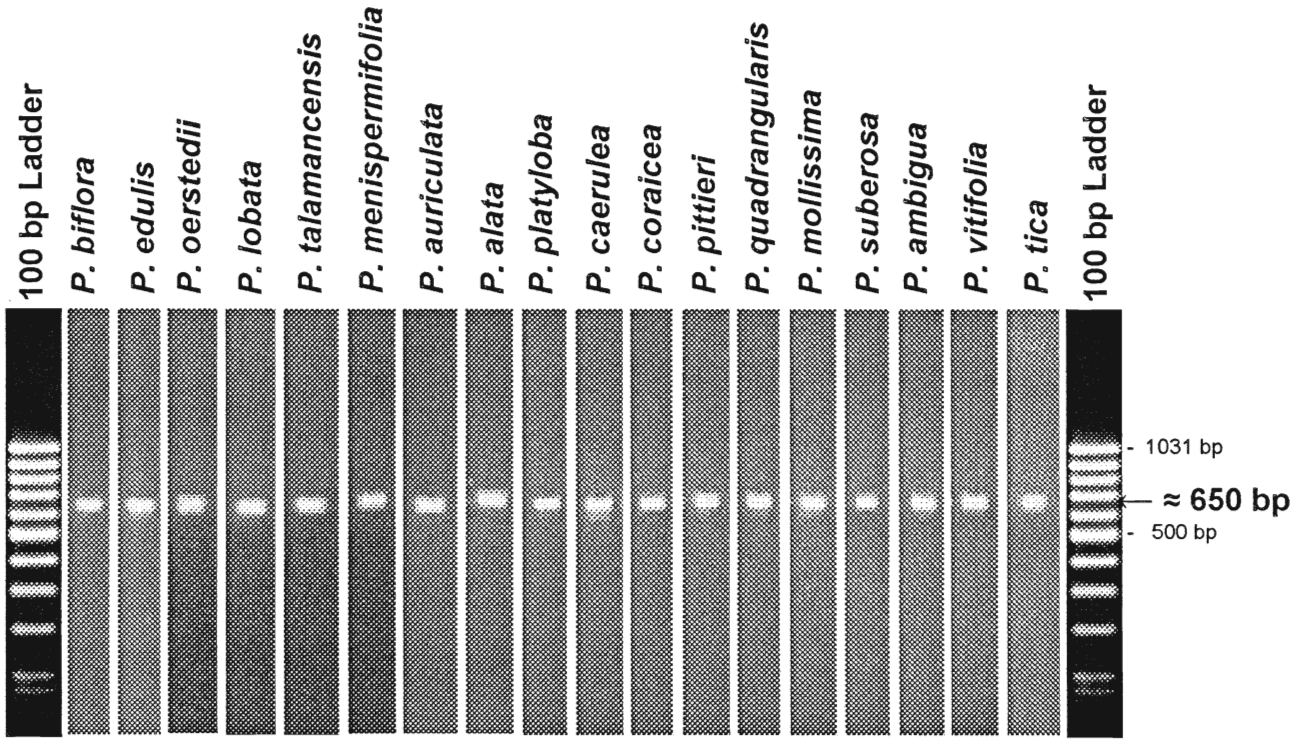

Figure 6. tRNA-Leucine intron PCR products from 18 species of *Passiflora* visualized on a 1% agarose gel stained with ethidium bromide. Far right and left lanes show the molecular weight marker (GeneRuler™ 100bp DNA Ladder) with base pair sizes indicated on the right of the figure. Number in bold with arrow indicates approximate size of bands.

Table 12. Lengths of sequences obtained from each of the primers for the two gene region examined in the 18 species of Passifloraceae.

Passifloraceae Species	W-ITS 1	W-ITS 2	W-ITS 3	W-ITS 4	ITS1/5.8S/ITS2 dsDNA	cB49317	dA49855	tRNA-Leucine Intron dsDNA
P. alata	671	288	394	671	673	626	629	625
P. ambigua	480	302	398	601	684	623	622	602
P. auriculata	570	339	377	686	700	612	618	594
P. biflora	602	343	370	620	686	619	622	620
P. caerulea	626	291	400	681	651	620	629	607
P. coriacea	692	339	366	691	705	620	630	610
P. edulis	560	285	383	668	671	624	628	606
P. lobata	691	338	365	699	687	605	401	614
*P. menispermifolia**	650	290	314	666	643	629	630	585
P. mollissima	n/a	n/a	n/a	n/a	n/a	588	624	614
P. oerstedii	660	287	590	667	890	628	572	600
P. pittieri	n/a	n/a	n/a	n/a	n/a	641	638	610
P. platyloba	590	295	386	490	671	622	638	624
P. quadrangularis	510	287	383	626	656	586	620	593
P. suberosa	426	335	369	694	696	565	532	561
P. talamancensis	420	330	365	632	652	622	623	601
P. tica	n/a	n/a	n/a	n/a	n/a	620	621	601
P. vitifolia	451	290	388	671	673	587	615	586

SEQUENCE ALIGNMENT AND CHARACTER SELECTION – *HELICONIUS*

Upon alignment of the ITS 2 sequences for the 14 heliconiine species, a region of approximately 400 base pairs of double stranded DNA (dsDNA) was obtained from which characters were obtained for phylogenetic analysis. Characters were selected only from the regions where the alignment was strong (see Appendix C for the ITS 2 alignment). A total of 68 informative characters were obtained from the strong alignment regions, five of which were selected to provide strength to the outgroup. Table 6 provides a summary of the characters utilized by alignment region. Where alignment regions were strong for the ingroup (*Heliconius/Laparus*) yet uncertain for the outgroups (including *A. vanillae*, *Dryadula phateusa* and *Dryas iulia*), only the ingroup characters were utilized as informative in the phylogenetic analysis. The 14 aligned heliconiine ITS 2 sequences were submitted to GenBank under Accessions AF453762 to AF453775. For the list of all Accession numbers see Appendix G.

The alignment of the partial EF-1α sequences for the 14 heliconiines resulted in a solid block of approximately 440 base pairs aligned from dsDNA. In *H. hortense*, *H. cydno* and *H. sara* a few base pairs conflicted for the forward and reverse sequences and therefore additional individuals were sequenced. For *H. hortense* and *H. sara* these conflicting base identities were resolved by the additional sequencing effort. However, for *H. cydno* a polymorphism was detected at site 177 with one individual having cytosine and another thymine for both forward and reverse sequences. This site was therefore coded for with the IUPAC symbol Y for cytosine and/or thymine (see Appendix D for the EF-1α alignment).

These sequences for all 14 species of heliconiines have been submitted to GenBank under Accessions AF454810 to AF454823. Table 7 lists the total number of characters

obtained from the partial EF-1α alignment region. In total, 45 informative characters were utilized with seven of these characters selected to strengthen the outgroup position of *A. vanillae*. In addition, as this is a protein coding region, the reading frame was deciphered and all characters were sorted as first, second or third position changes. These changes are listed in Table 10. The majority of changes (38) were third position silent changes. However, two changes resulted in a change in the amino acid sequence. At site 234 for *L. doris* and *H. hortense* a second position change results in the amino acid changing from lysine to arginine. Also, at site 424 a third position change in the ingroup (excluding *A. vanillae*, *Dryadula phateusa* and *Dryas iulia*) has resulted in a change from glutamic acid to aspartic acid (see Table 10).

SEQUENCE ALIGNMENT AND CHARACTER SELECTION – *PASSIFLORA*

The alignment of the ITS 1/5.8S/ITS 2 region of the *Passiflora* species was completed with the outgroup *Sphaerocardamum nesliiforme* sequence obtained from GenBank (Accession number AF055195) (Bailey and Doyle, 1999). Prior to the alignment of dsDNA for all species combined, the alignment of the four sequences from the four primers for each species combined was performed. In *P. pittieri* and *P. tica*, sequence alignment was impossible due to poor sequencing results. Therefore, the ITS 1/5.8S/ITS 2 alignment was performed for only 15 species of *Passiflora* (see Appendix E for the ITS 1/5.8S/ITS 2 alignment). These 15 aligned sequences have been submitted to GenBank under Accession numbers AF454795 to AF454809.

The complete alignment region for these 15 species consisted of approximately 640 base pairs of dsDNA. In total, 99 of these were informative characters as can be seen in

Table 8. As with the ITS 2 region for the heliconiines, only characters within regions of strong alignment were utilized for phylogenetic analysis. Also, where the *Passiflora* ingroup could not be aligned confidently with the *S. nesliiforme* outgroup sequence, the outgroup sequence was removed and only ingroup characters were used.

The second *Passiflora* gene region examined, the tRNA-Leucine intron, resulted in an alignment of approximately 580 base pairs for all 17 species (see Appendix F). The outgroup sequence used in the tRNA-Leucine intron alignment was *S. nesliiforme* (Accession AF055263) obtained from GenBank (Bailey and Doyle, 1999). In total, 67 informative characters were utilized in phylogenetic analysis from the tRNA-Leucine intron alignment. This included two gap characters. To accommodate the gap characters, the data matrix was converted to numbers in MacClade. As with the ITS 1/5.8S/ITS 2 alignment, the outgroup sequence was removed in regions where it did not align confidently with the *Passiflora* ingroup (see Table 9) and only ingroup characters were included. All 18 sequences from the tRNA-Leucine intron alignment have been submitted to GenBank under Accessions AF454778 to AF454794 and AF461415.

All four data sets were found to contain significant phylogenetic information. For each of the four gene regions, the g1 skew values were below the critical values for significance at the $P<0.01$ level (see Table 13) (Hillis and Huelsenbeck, 1992).

Table 13. Measures of skew (g1) in tree length distribution from 1000 randomly generated trees for each data set from Heliconiinae and Passifloraceae.

Family	Data Set	Skew (g1)
Heliconiinae	ITS 2	-0.7342
	EF-1α	-0.9797
Passifloraceae	tRNA-Leucine	-0.5575
	ITS 1/5.8S/ ITS 2	-0.6889

PHYLOGENETIC ANALYSIS USING PARSIMONY – *HELICONIUS*

Individual analysis of the ITS 2 data yielded four equally parsimonious trees (EPT) with a treelength of 111, a CI of 0.82 and a RI of 0.87 which could not be reduced by reweighting the characters. Amongst the four trees the 'silvaniform-melpomene' group is resolved in Trees 2 and 4 and not entirely resolved in Trees 1 and 3 with the collapse of *H. cydno* and *H. ismenius*. Also, the position of *H. hortense* is equivocal as either ancestral to *H. charithonia* in Trees 1 and 3 or ancestral to the entire group in Trees 2 and 4. As such, Tree 3 (Figure 7) was selected as it most closely resembles the 50% consensus tree. The *Heliconius* / *Laparus* ingroup has a bootstrap support of 100 as does the 'silvaniform-melpomene' group. The 'sara-sapho' and 'charithonia' clade is also strongly supported with a bootstrap value of 99.

Phylogenetic analysis of the partial EF-1α sequence data resulted in three EPT. Upon reweighting of the characters, one tree was derived with a treelength of 104, a CI of 0.73 and a RI of 0.76 (Figure 8). Bootstrap values provide strong support for the *Heliconius* / *Laparus* ingroup (bootstrap=100) and the 'silvaniform-melpomene' clade (bootstrap=100).
The two data sets for the 14 species of Heliconiinae were found to be the same (partition homogeneity test, P=0.81) and therefore, the data sets were combined. With the combination of the EF-1α and ITS 2 data matrices, phylogenetic analysis derived only one tree (Figure 9) with a treelength of 220, a CI of 0.76 and a RI of 0.81. In this phylogeny, bootstrap values and Bremer Decay Indices give strong support to the *Heliconius* / *Laparus* ingroup (bootstrap=100; BDI=18) as well as the 'silvaniform-melpomene' clade (bootstrap=100; BDI=16). However, the placement of *H. cydno* with *H. ismenius* and

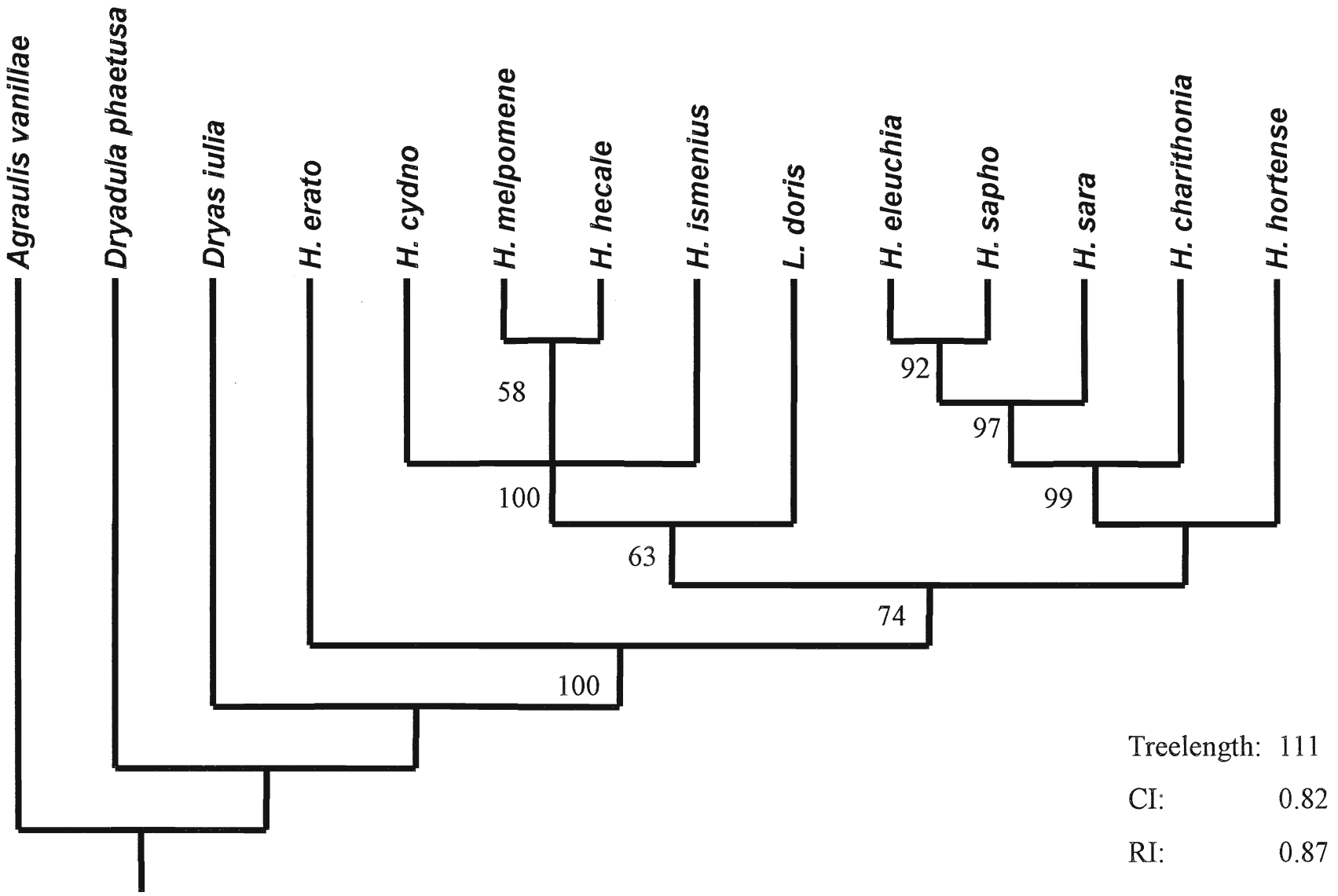

Figure 7. *Heliconius* phylogeny based on ITS 2 sequence data. Numbers on the left side of nodes represent bootstrap percent values.

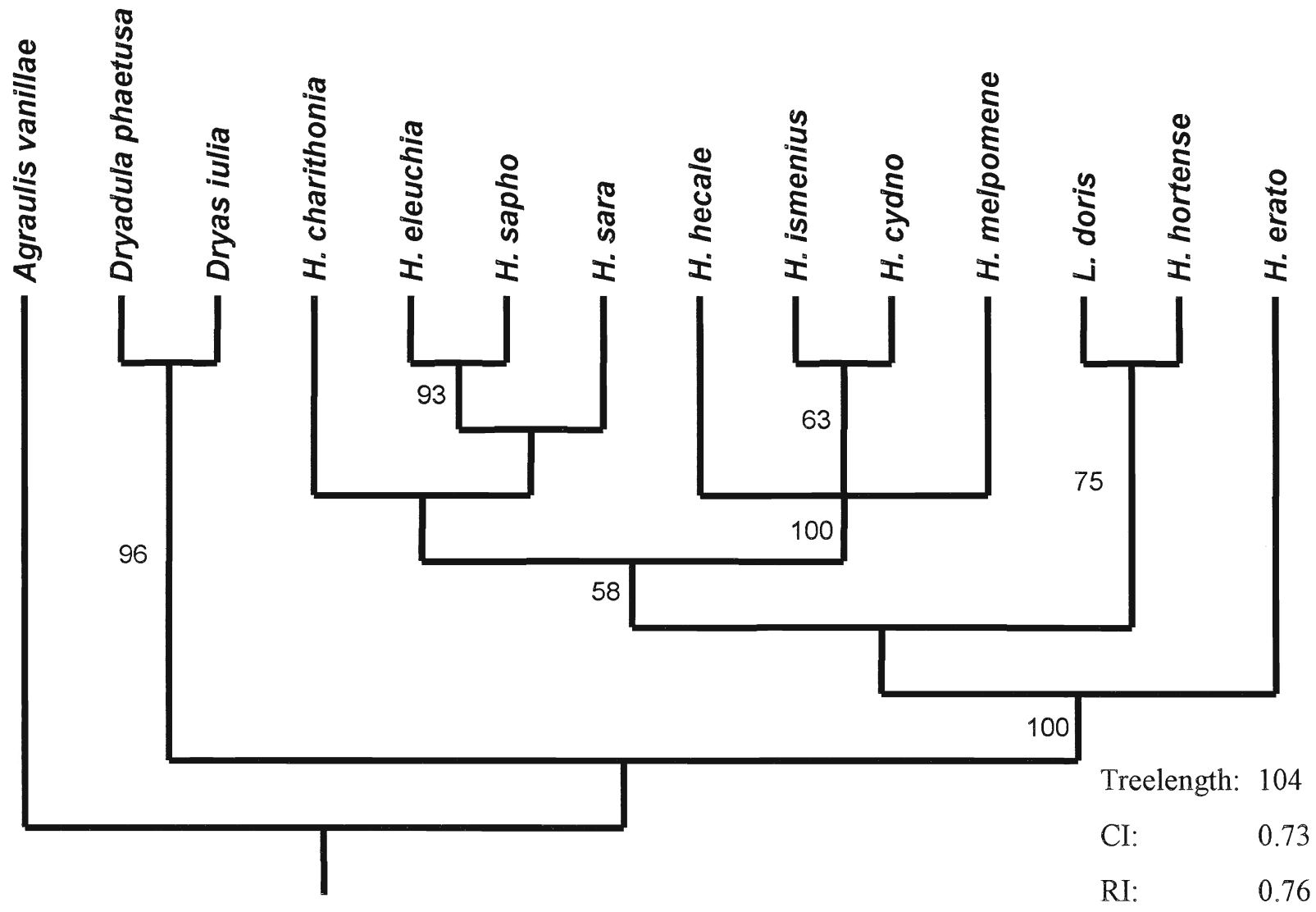

Figure 8. *Heliconius* phylogeny based on partial EF-1α sequence data. Numbers on the left side of nodes represent bootstrap percent values.

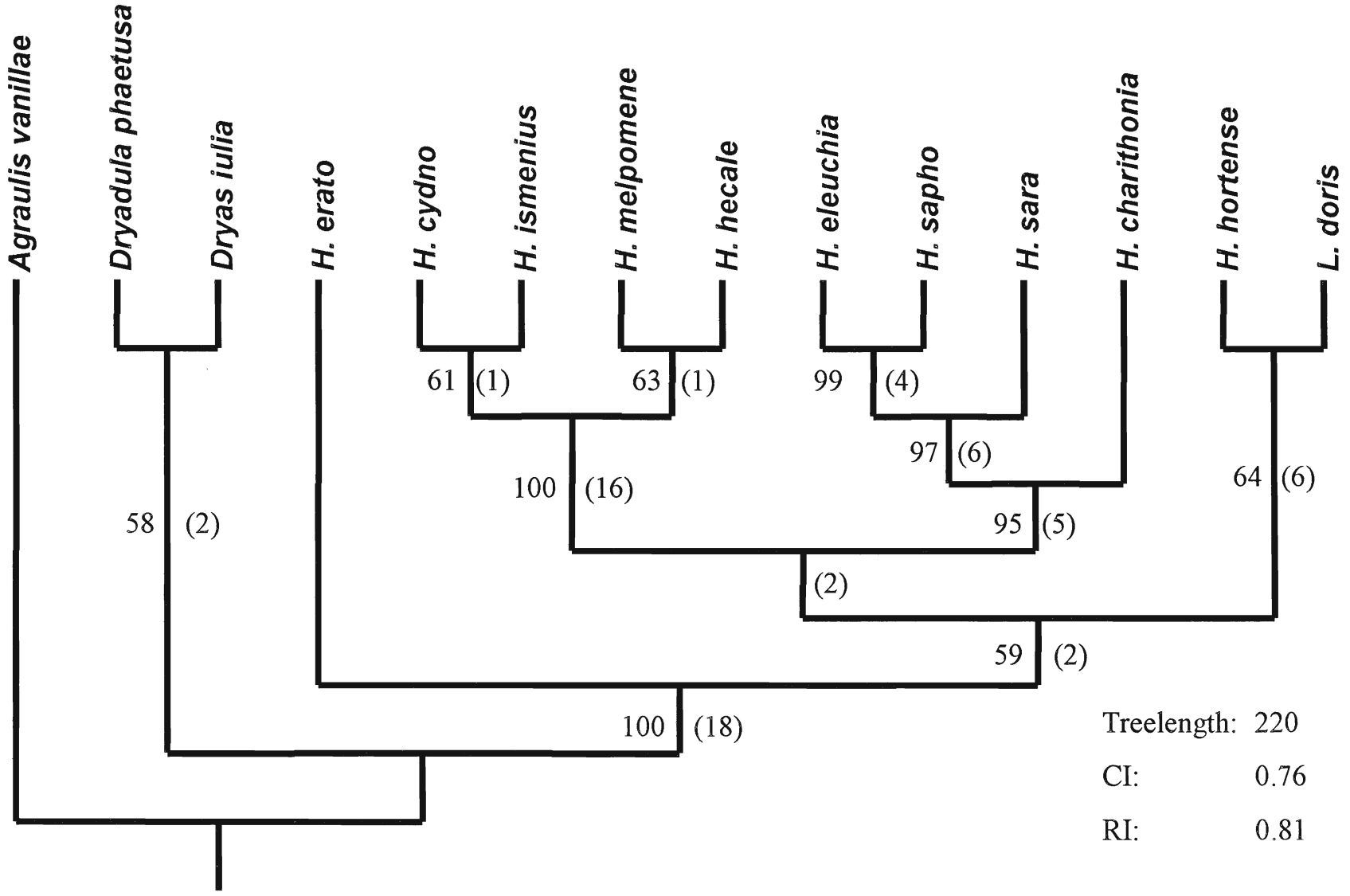

Figure 9. *Heliconius* phylogeny based on partial EF-1α and ITS 2 sequence data combined. Numbers on the left side of nodes represent bootstrap percent values and numbers in parentheses (right of nodes) represent strength of grouping by Bremer Decay Indices.

H. melpomene with *H. hecale* as sister taxa is not strongly supported (bootstrap=61 and 63 respectively; BDI=1 for both groups). The 'sara-sapho' and 'charithonia' clade is also well supported with a bootstrap value of 95 and BDI of 5. Although these clades are supported by the combined data, the placement of the ingroup ancestral heliconiines is not well supported. For example, the placement of *H. erato* as ancestral to the remaining *Heliconius/Laparus* taxa received a low bootstrap support of 59 and BDI of 2. Similarly, the most ancestral group *L. doris* and *H. hortense* had no bootstrap support and collapsed with only a 2 step increase in treelength (BDI of 2). Also, within the outgroup, the ancestral Heliconiinae, *Dryadula phaetusa* and *Dryas iulia,* are only weakly supported as sister groups (bootstrap=58; BDI=2). This arrangement is not well supported due to the conflicting results of each data set analysis individually. For example, the EF-1α data strongly supports these two taxa as sisters (bootstrap=96) whereas the ITS 2 data finds *Dryadula phaetusa* to be more ancestral than *Dryas iulia*, although this topology was not supported by bootstrapping.

PHYLOGENETIC ANALYSIS USING PARSIMONY – *PASSIFLORA*

Figure 10 represents the selected phylogeny of the 15 species of *Passiflora* based on the ITS 1/5.8S/ITS 2 data. Initially the analysis of this data set resulted in five EPT and upon reweighting the data, two EPT were derived with a treelength of 167, a CI of 0.75 and a RI of 0.84. Here, two clades are distinctly supported. These two clades represent the *Passiflora* (*Granadilla*) group (bootstrap=99) and the *Decaloba* (*Plectostemma*) group (bootstrap=100). The one difference between the two final tree topologies was the equivocal placement of *P. auriculata* as either ancestral to the whole *Decaloba* (*Plectostemma*) clade or ancestral to the

P. biflora, *P. talamancensis* and *P. lobata* group only. Although Tree 1 was selected, either topology is equally possible.

The analysis of the tRNA-Leucine intron data resulted in the tree shown in Figure 11. This topology was derived from reweighting the original analysis which initially resulted in two EPT. The treelength of this tree is 86, the CI is 0.91 and the RI is 0.93 and its topology is the same as Tree 1 in the original two trees. Although the tRNA-Leucine intron data do display polytomies, a number of the groups are well supported with bootstrapping. As with the ITS 1/5.8S/ITS 2 data, the two major clades *Passiflora* (*Granadilla*) and *Decaloba* (*Plectostemma*) are well supported both with a bootstrap value of 96. The two additional taxa included in this analysis, *P. pittieri* and *P. tica*, which represent the subgenus *Astrophea*, are also well supported as a group together (bootstrap=94). The *Astrophea* group was found to be ancestral to the *Passiflora* (*Granadilla*) group although bootstrapping did not support this well (bootstrap=58).

The two data sets for the 17 species of Passifloraecae were found to be the same (partition homogeneity test, P=1.00) and these data sets were therefore able to be combined. In the phylogenetic analysis of both *Passiflora* data sets combined, the initial result was three EPT. When the data were reweighted, two EPT were derived. Both of these trees are fully resolved and have a treelength of 257, a CI of 0.80 and a RI of 0.87. As with the ITS 1/5.8S/ITS 2 phylogeny results, the position of *P. auriculata* is equivocal between the two final trees. Although Tree 1 has been selected here, either tree is equally likely. Figure 12 shows Tree 1: The selected phylogeny of the 17 species of *Passiflora* based on tRNA-Leucine intron and ITS 1/5.8S/ITS 2 gene data.

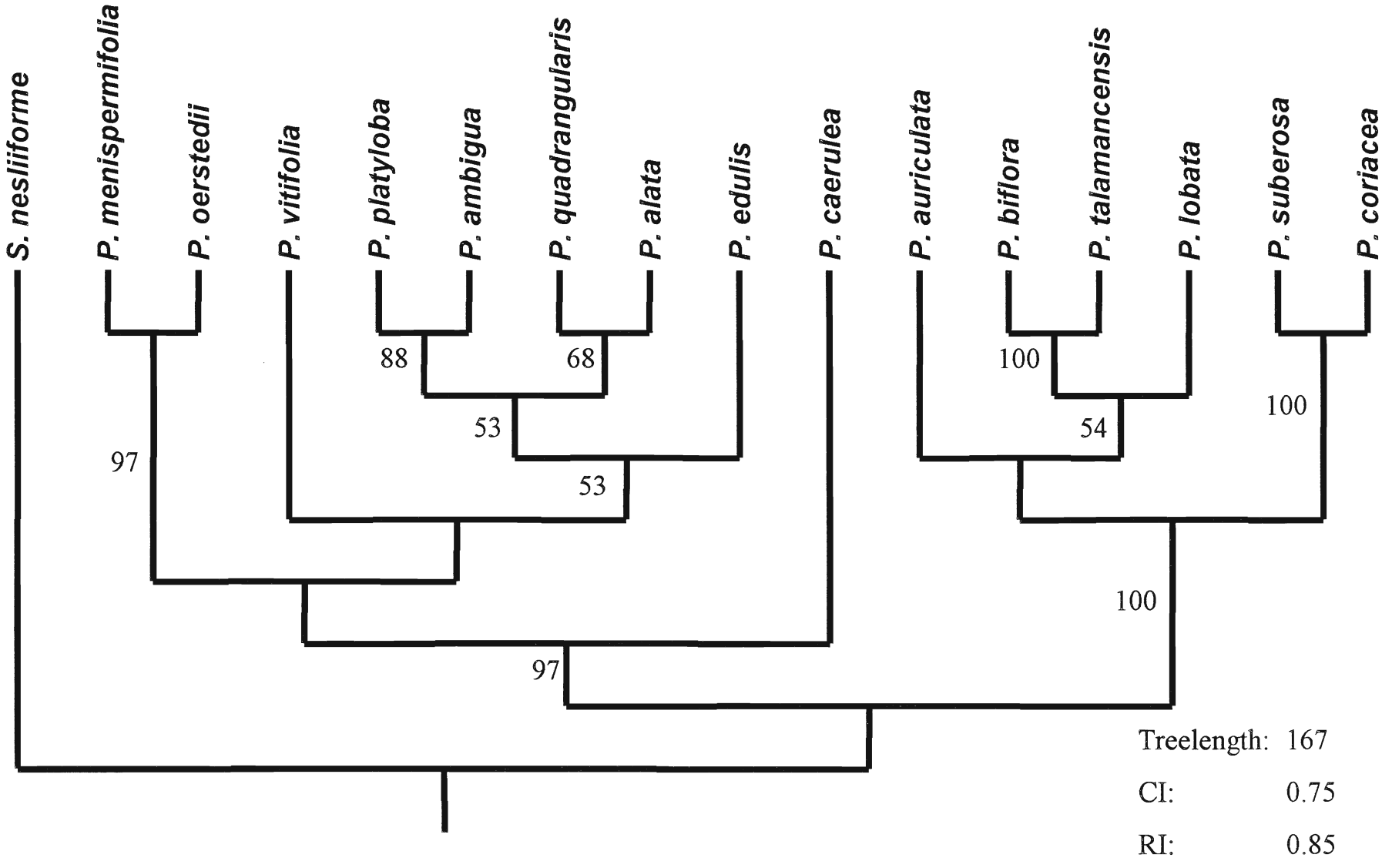

Figure 10. *Passiflora* phylogeny based on ITS1/5.8S/ITS2 sequence data. Numbers on the left side of nodes represent bootstrap percent values.

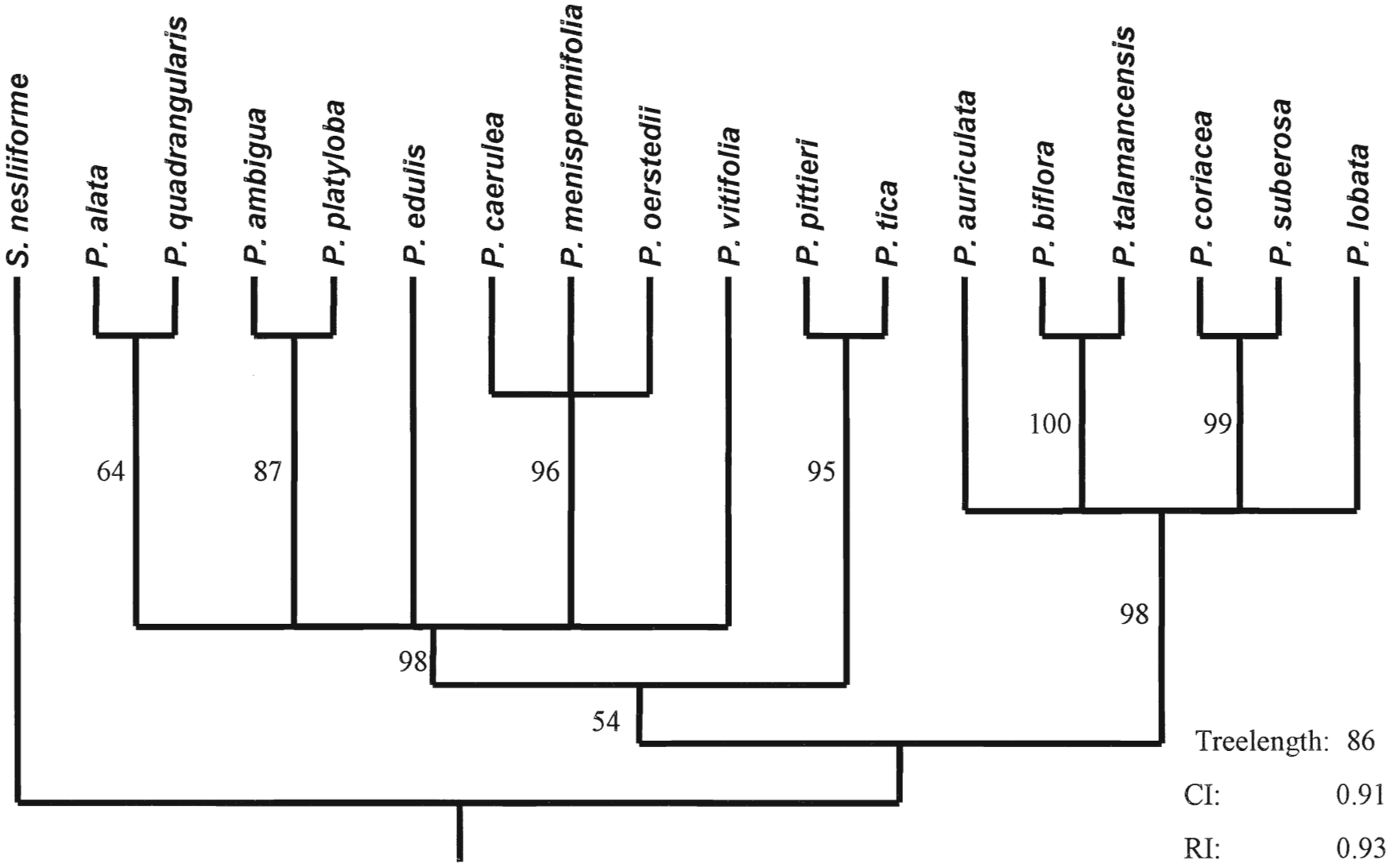

Figure 11. *Passiflor*a phylogeny based on *trn*L sequence data. Numbers on the left side of nodes represent bootstrap percent values.

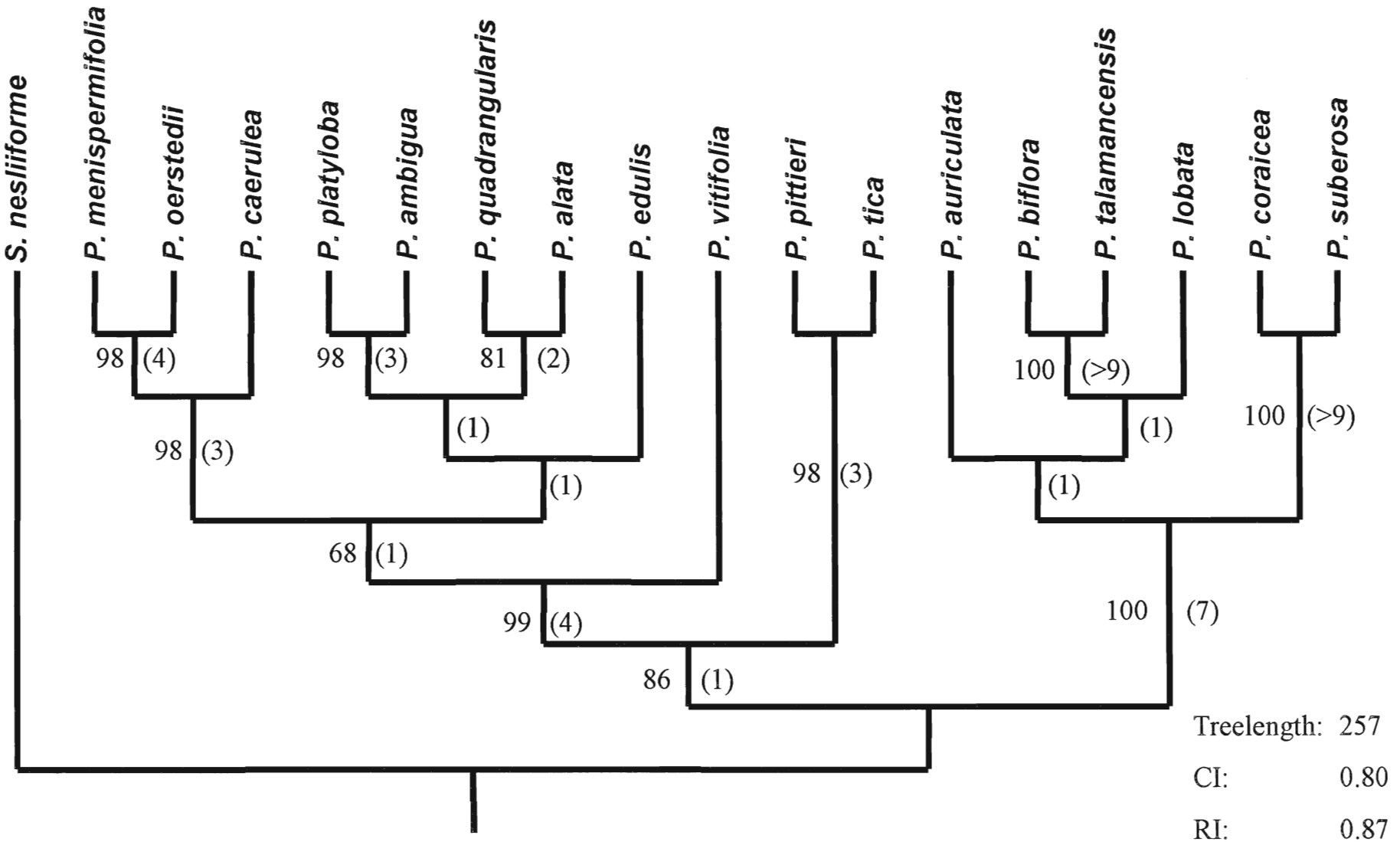

Figure 12. *Passiflora* phylogeny based on ITS1/5.8S/ITS2 and *trn*L sequence data combined. Numbers on the left side of nodes represent bootstrap percent values and numbers in parentheses (right of nodes) represent strength of grouping by Bremer Decay Indices.

In this final phylogeny the two main clades, *Passiflora* (*Granadilla*) and *Decaloba* (*Plectostemma*) are well supported with bootstrapping (99 and 100 respectively). The BDI for each of these clades shows differential support. While the *Decaloba* (*Plectostemma*) clade is strongly supported (BDI=7), the *Passiflora* (*Granadilla*) group is not well supported by the BDI with the internal branch collapsing after only 1 step increase in treelength. This clade overall however, including the *Astrophea* subgenus, is supported with a BDI of 4 and bootstrap value of 86.

Within each of these two major groups, a number of the species relationships at the tree tips received strong support. Within *Passiflora* (*Granadilla*), the group *P. menispermifolia*, *P. oerstedii* and *P. caerulea* as well as the species pairs *P. menispermifolia* with *P. oerstedii*, *P. platyloba* with *P. ambigua* and *P. quadrangularis* with *P. alata* are relatively well supported (see Figure 12). The placement of the two *Astrophea* species, *P. pittieri* and *P. tica,* as sister taxa is also well supported. Finally, within the *Decaloba* (*Plectostemma*) subgenus the pairing of *P. biflora* with *P. talamancensis* and *P. coriacea* with *P. suberosa* is very strongly supported, although the internal nodes are weak within this clade due to the equivocal position of *P. auriculata.*

PHYLOGENETIC ANALYSIS USING MAXIMUM LIKELIHOOD

The statistical results from the maximum likelihood analysis including base frequencies, ln(L) and Ti/Tv for each of the four gene regions are listed in Table 14. The tree obtained from the likelihood analysis for the ITS 2 sequence data is presented in Figure 13. This topology is almost identical to that achieved from the parsimony analysis (Figure 7). The one difference in the likelihood tree is the placement of *H. hortense* as ancestral to

the two internal heliconiine clades with *L. doris* as opposed to ancestral within the 'charithonia-sapho-sara' clade. When the parsimony topology was changed to this likelihood topology using MacClade, the treelength remained the same as did the CI and RI values.

The maximum likelihood representation of the partial EF-1α sequence data is shown in Figure 14. There are two main differences between this topology and that seen in Figure 8 (the parsimony tree). Rather than the two internal derived sister clades seen in the parsimony tree, the likelihood tree has *H. charithonia*, *H. sapho* and *H. sara* as ancestral to the remainder of the heliconiines. The second major difference is the placement of *H. ismenius* and *H. cydno* as ancestral in the likelihood tree as compared to derived in the parsimony topology. When the parsimony tree was changed in MacClade to the maximum likelihood topology, the treelength was increased from 104 to 114 with the CI decreasing to 0.67 and the RI to 0.68.

The tree derived from the maximum likelihood analysis of the tRNA-Leucine sequence data is shown in Figure 15. In comparison to the parsimony tree (Figure 11), this tree has a slightly different arrangement of the *Passiflora*, *Decaloba* and *Astrophea* subgenera. Firstly, within the Passiflora group, *P. ambigua* and *P. platyloba* are ancestral. Secondly, *P. auriculata* is ancestral in the *Decaloba* group and, thirdly, the *Astrophea* group (*P. pittieri* and *P. tica*) is the most ancestral group. As these differences are only minor, the treelength is only increased by one with the alteration of the parsimony tree to the likelihood form (CI=0.90; RI=0.92).

The maximum likelihood tree for the ITS 1/ 5.8S/ITS 2 data for the *Passiflora* species is shown in Figure 16. This phylogenetic representation of these taxa is somewhat

Table 14. Empirical Nucleotide Frequencies, Best Tree Scores and Estimated Transition / Transversion Ratio for sequence data from Heliconiinae and Passifloraceae.

	Gene Region	Empirical Nucleotide Frequencies				Best Tree Score (ln(L))	Estimated Ti/Tv Ratio
Heliconiinae	**ITS 2**						
		A	**C**	**G**	**T**		
		0.2868	0.1628	0.2734	0.277	-512.40	0.95
	EF-1α						
		A	**C**	**G**	**T**		
		0.1429	0.3675	0.1127	0.377	-300.80	15.61
Passifloraceae	**ITS 1/5.8S/ITS 2**						
		A	**C**	**G**	**T**		
		0.2805	0.2924	0.2328	0.1943	-755.18	
	tRNA-Leucine						
		A	**C**	**G**	**T**		
		0.2813	0.2875	0.1656	0.2656	-377.02	1.01

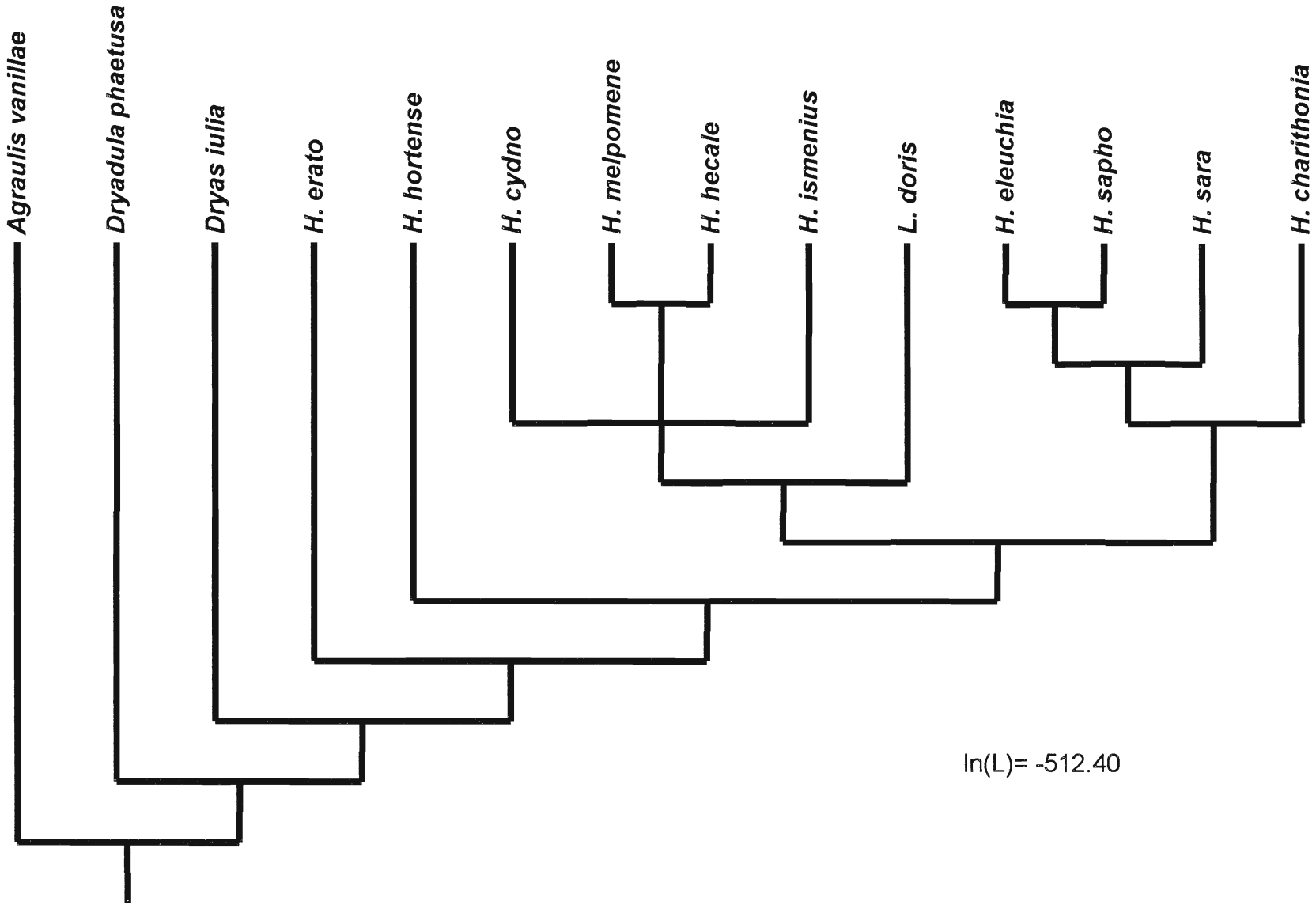

Figure 13. Phylogeny of ITS 2 sequence data from 14 species of Heliconiinae using Maximum Likelihood analysis.

Figure 14. Phylogeny of partial EF-1α sequence data from 14 species of Heliconiinae using Maximum Likelihood analysis.

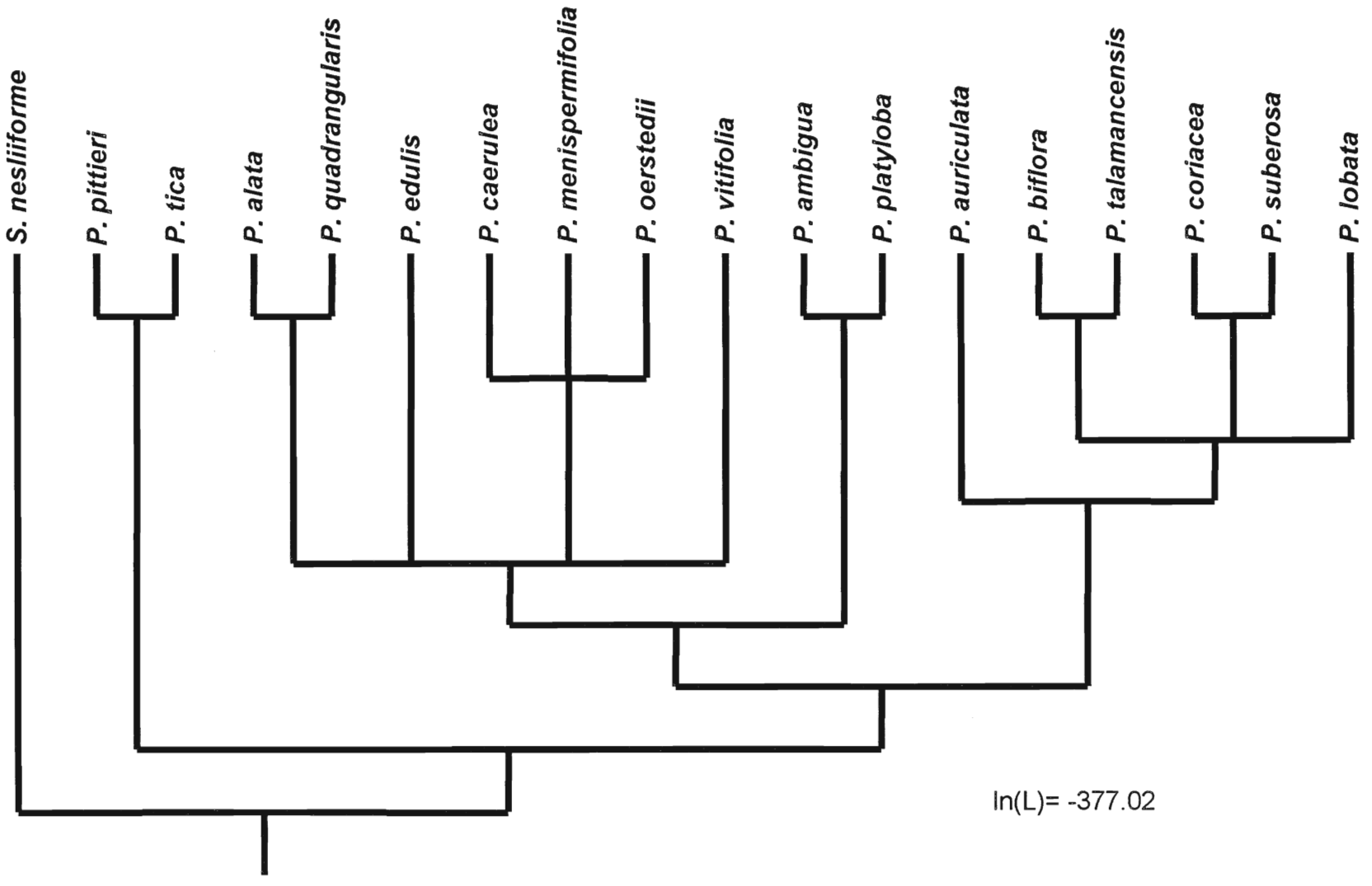

Figure 15. Phylogeny of tRNA-Leucine sequence data of 17 species of *Passiflora* using Maximum Likelihood analysis.

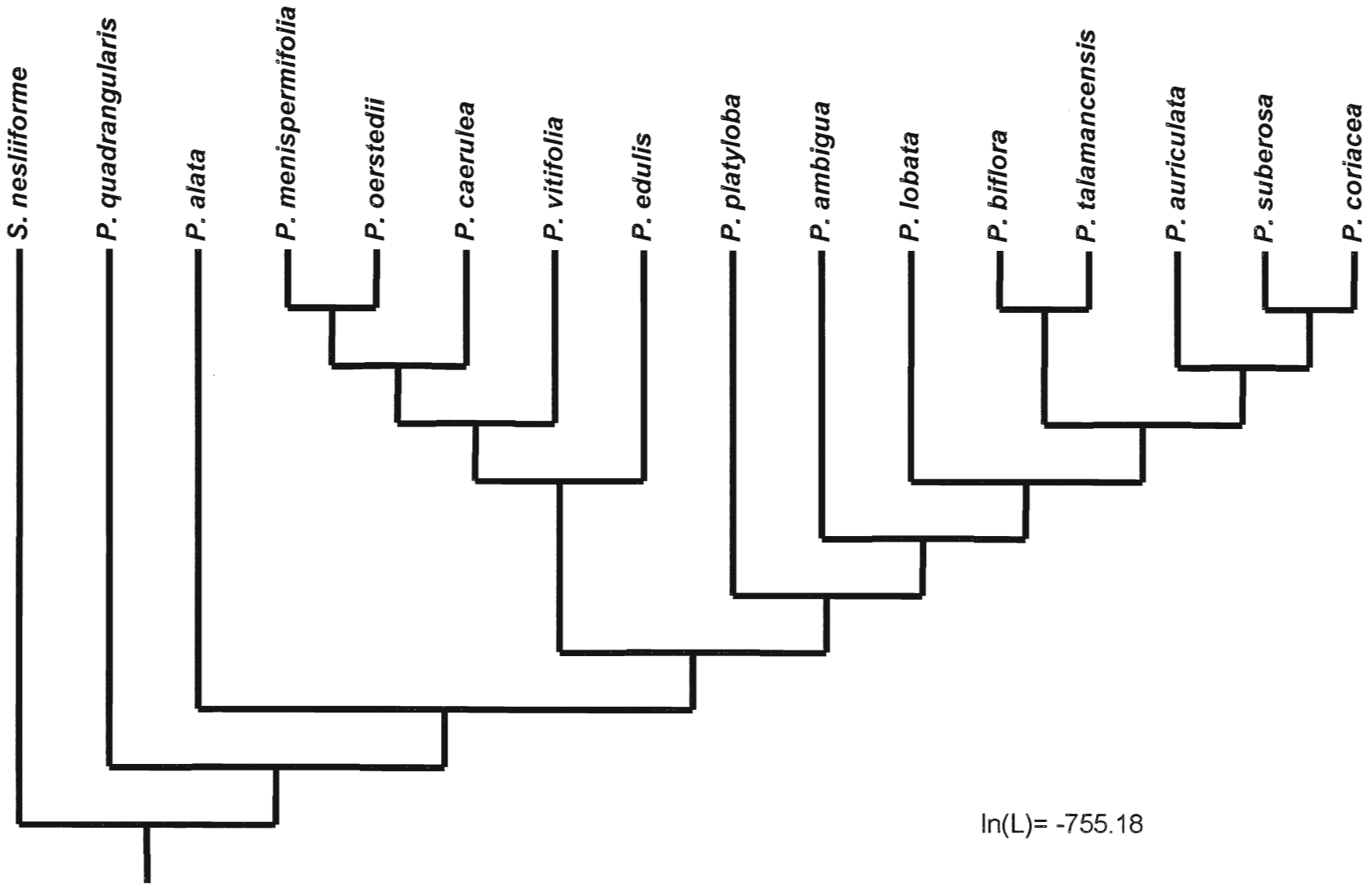

Figure 16. Phylogeny of ITS 1/5.8S/ITS 2 sequence data from 17 species of *Passiflora* using Maximum Likelihood analysis.

different from that derived from the parsimony analysis. Firstly, although the *Decaloba* subgenus remained intact, the arrangement of the taxa differs with *P. lobata* ancestral. However, ancestral in this clade are *P. ambigua* and *P. platyloba* respectively, which, in the parsimony phylogeny (Figure 10) are derived within the *Passiflora* subgenus. Thirdly, the more ancestral of the *Passiflora* group in the parsimony topology form a sister clade to the aforementioned clade in the maximum likelihood tree. Lastly, the greatest difference in the likelihood representation is the placement of *P. quadrangularis* and *P. alata* as ancestral amongst the entire *Passiflora* subgenus. This contradicts the highly derived position in the parsimony tree (see Figure 10). The changing of the parsimony tree to the likelihood topology in MacClade resulted in an increased treelength of 170 (from 167) and a slightly decreased CI (0.74) and RI (0.83) (from 0.75 and 0.85, respectively).

PHYLOGENETIC COMPARISON FOR CONGRUENCE – *HELICONIUS/PASSIFLORA*

The comparison of the Heliconiinae and *Passiflora* phylogenies for congruence was performed by topological comparison and host cladogram analysis. For both comparisons, the Heliconiinae phylogeny was changed to the topology in Figure 17. In this phylogeny, *H. hortense* has been removed as it is not one of the species that was utilized in the phylogenetic comparison with *Passiflora*. The removal of this taxon resulted in the treelength being reduced to 210.

Further to this alteration, the phylogeny in Figure 17 has also been changed to represent the heliconiine phylogeny established in this study to the topology of Brower and Egan (1997). This alteration in the phylogenetic placement of these taxa has resulted in only a 1.4% increase in treelength to 213 (see Figure 17). As such, the phylogenetic

representation of the heliconiines presented here is a total evidence phylogeny and follows the Brower and Egan (1997) topology. Figure 17 also summarizes the Heliconiinae phylogeny divided up according to classification, feeding groups, pupal vs. non-pupal mating species and pollen feeding species as specified by Benson *et al.* (1975), Brown (1981) Gilbert (1983), Gilbert (1991) and Brower (1997). The *Passiflora* phylogeny with the species marked according to their classification by subgenus is shown in Figure 18.

The topological comparison to the butterfly and host plant phylogenies is seen in Figure 19. These results show little congruence between the two phylogenies with a high degree of crossover and host plant sharing. However, when the two main host plant groupings of *Passiflora* (*Granadilla*) + *Distephana* and *Decaloba* (*Plectostemma*) + *Astrophea* are separated, some matching can be seen (see Figure 20 and 21). These subgroups were made with *P. vitifolia* ancestral in the *Passiflora* (*Granadilla*)) + *Distephana* clade and the *Astrophea* group ancestral in the *Decaloba* (*Plectostemma*) group. Although the *Passiflora* (*Granadilla*)) + *Distephana* feeding group (Figure 20) does not show congruence, the *Decaloba* (*Plectostemma*) + *Astrophea* feeding groups shows some congruence (see Figure 21).

The second phylogenetic comparison that was performed was the host cladogram analysis. The data matrices for the host cladogram analysis of the complete set of Passifloraceae host plants are listed in Tables 15 and 16. As can be seen, the *Passiflora* (*Granadilla*) + *Distephana* feeding group and the *Decaloba* (*Plectostemma*) + *Astrophea* feeding groups were separated as in the topological comparison. The phylogenetic results of these data matrices are shown in Figure 22. The *Passiflora* (*Granadilla*) + *Distephana* host cladograms do not agree with the actual *Passiflora* phylogeny in topology. The *Decaloba*

(*Plectostemma*) + *Astrophea* host cladogram however, closely resembles the actual *Passiflora* phylogeny (Figure 22). One difference is the placement of *P. coriacea* within the *P. biflora* and *P. talamancensis* group. This represents the only major difference as the ancestral position of *P. auriculata* was already equivocal in the original two phylogenies derived for *Passiflora* based on the tRNA-Leucine intron and ITS 1/5.8S/ITS 2 gene data.

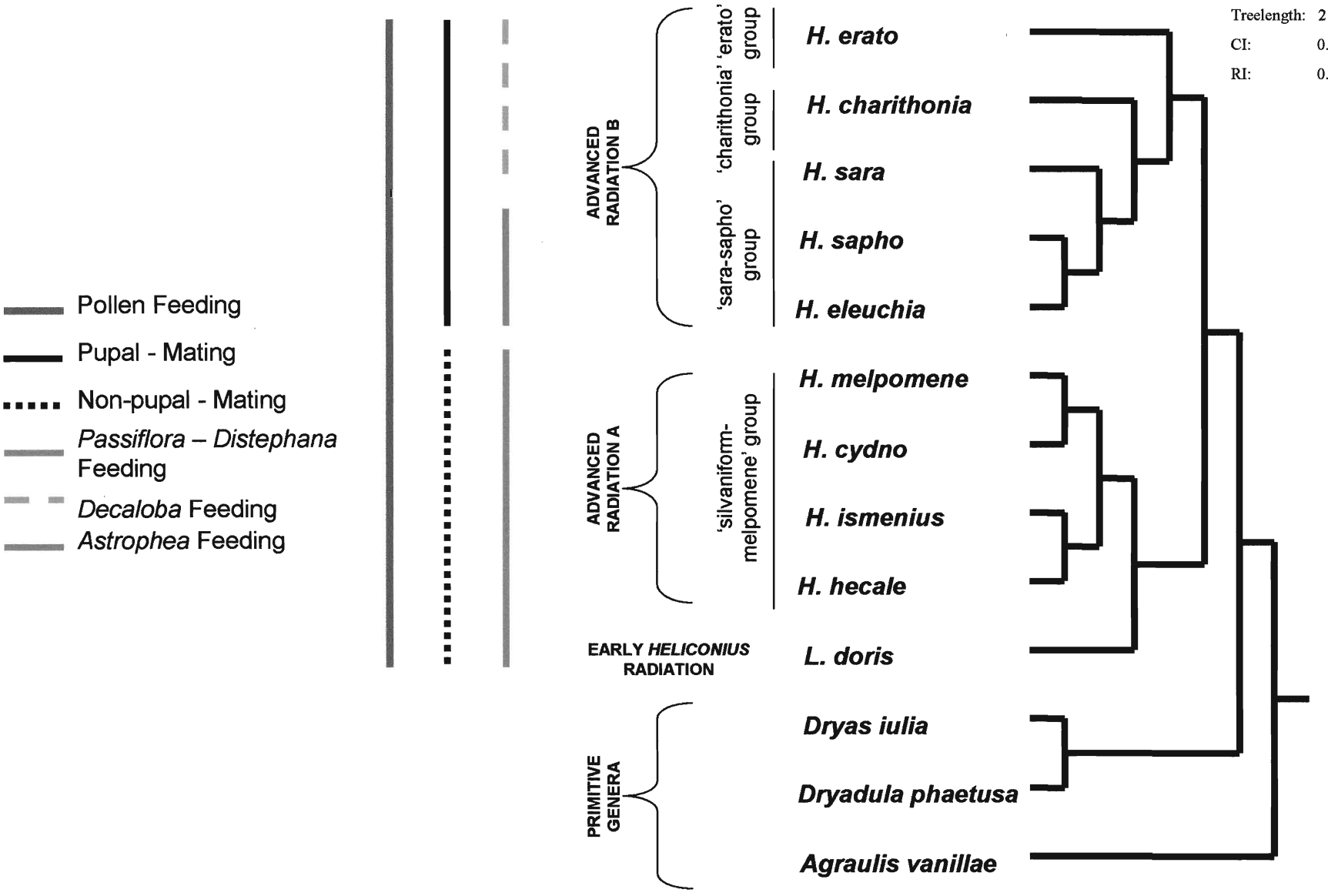

Figure 17. Phylogeny of 14 heliconiine species (based on ITS 2 and EF-1α sequence data) with sub-groupings as specified by Benson *et al.* (1975)

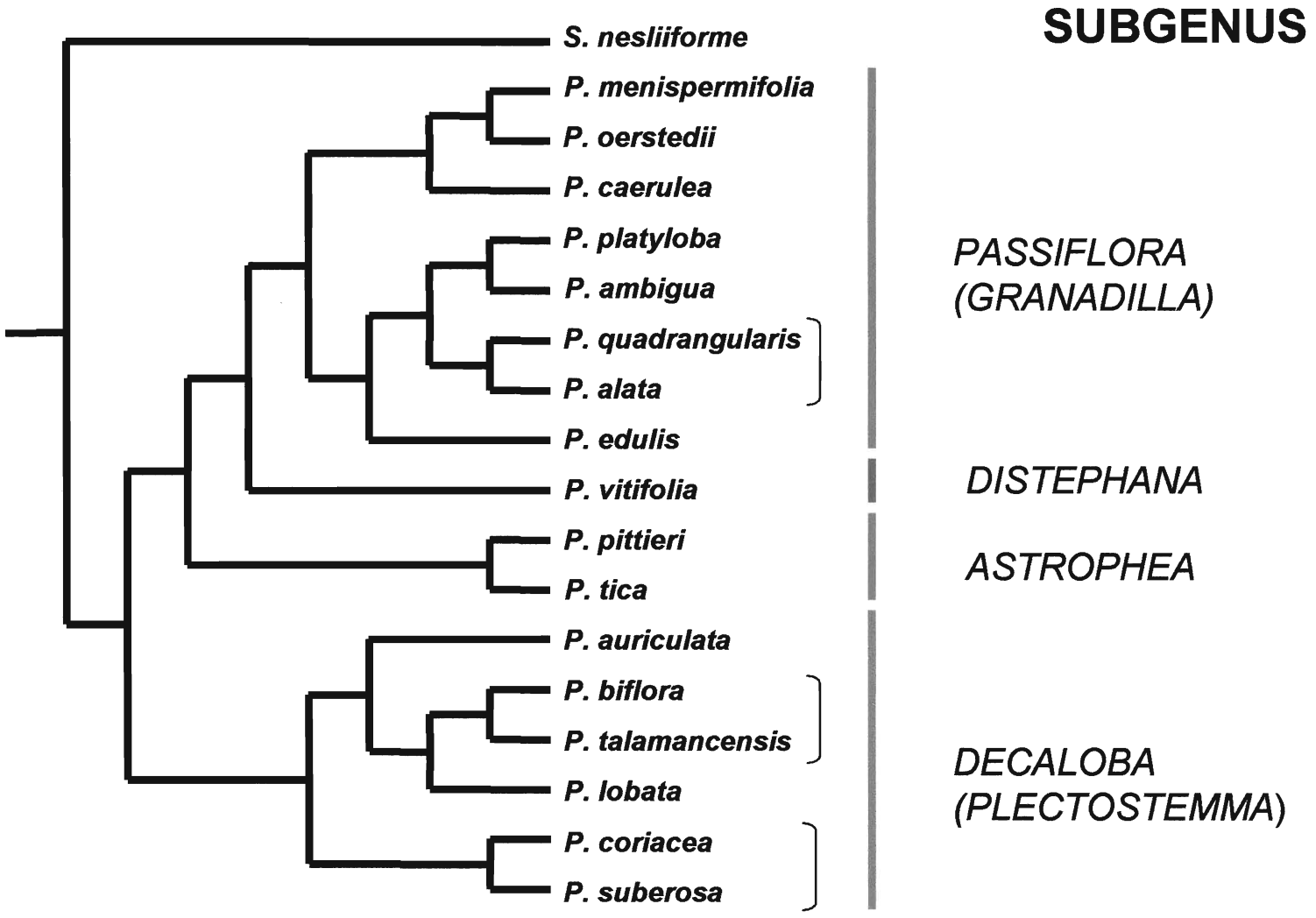

Figure 18. *Passiflora* phylogeny with classification by subgenus indicated. Short brackets show further subgroupings to Section and Series level (see Table 1).

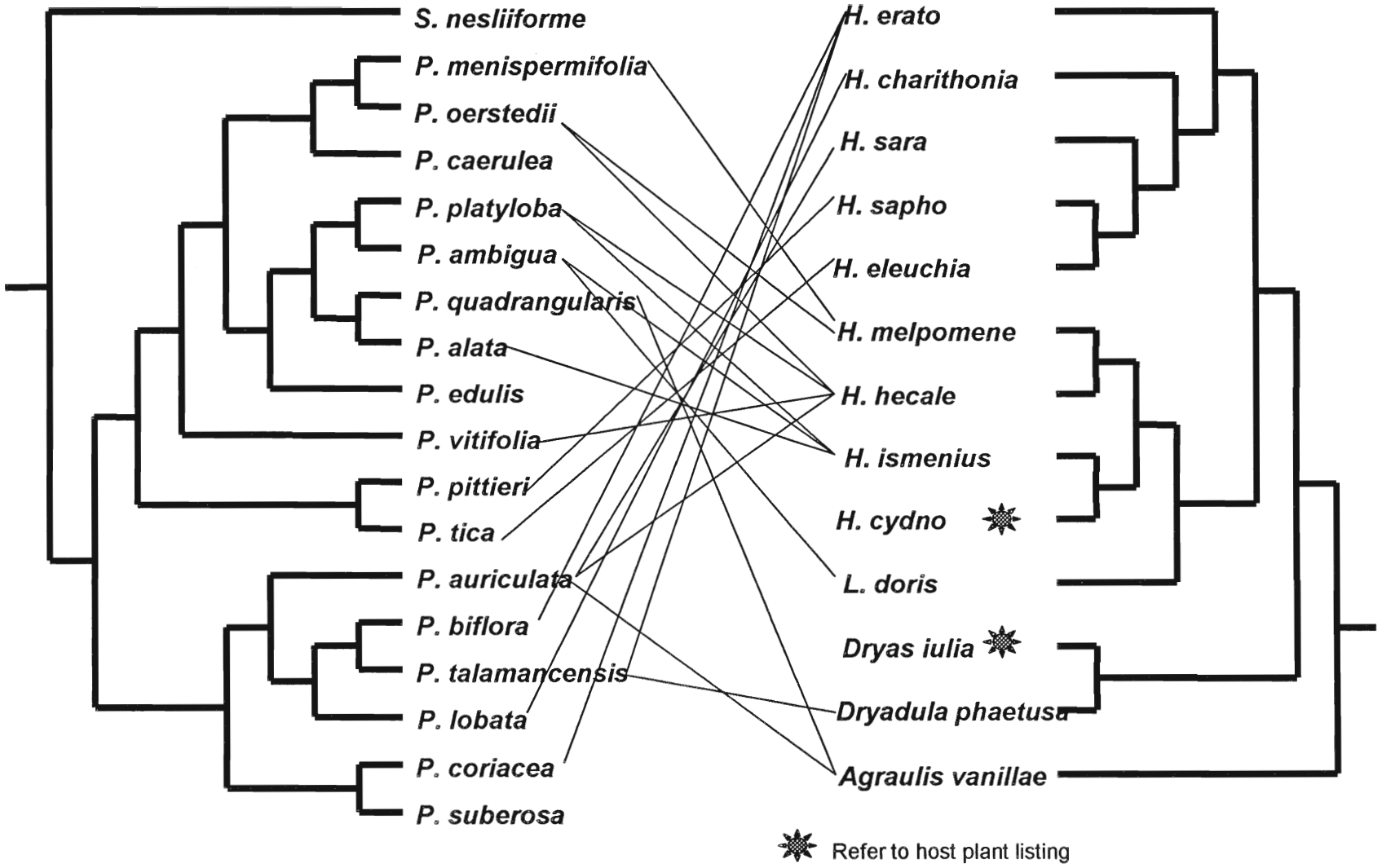

Figure 19. Comparison of the host *Passiflora* phylogeny (left) with the insect *Heliconius* phylogeny (right). Stars indicate generalist *Passiflora* feeders (see Table 2).

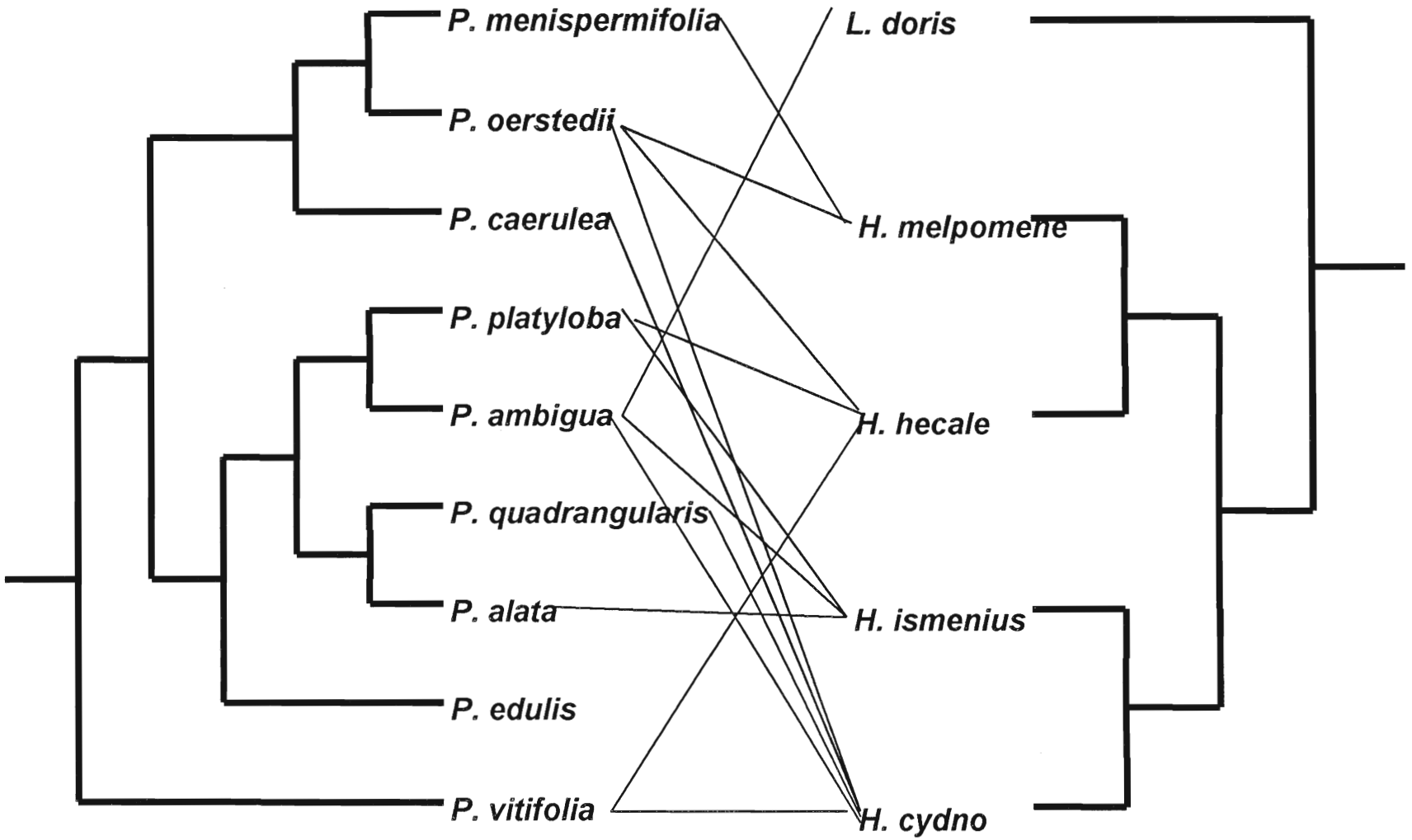

Figure 20. *Passiflora* (*Granadilla*) - *Distephana* Feeding Group by topological comparison.

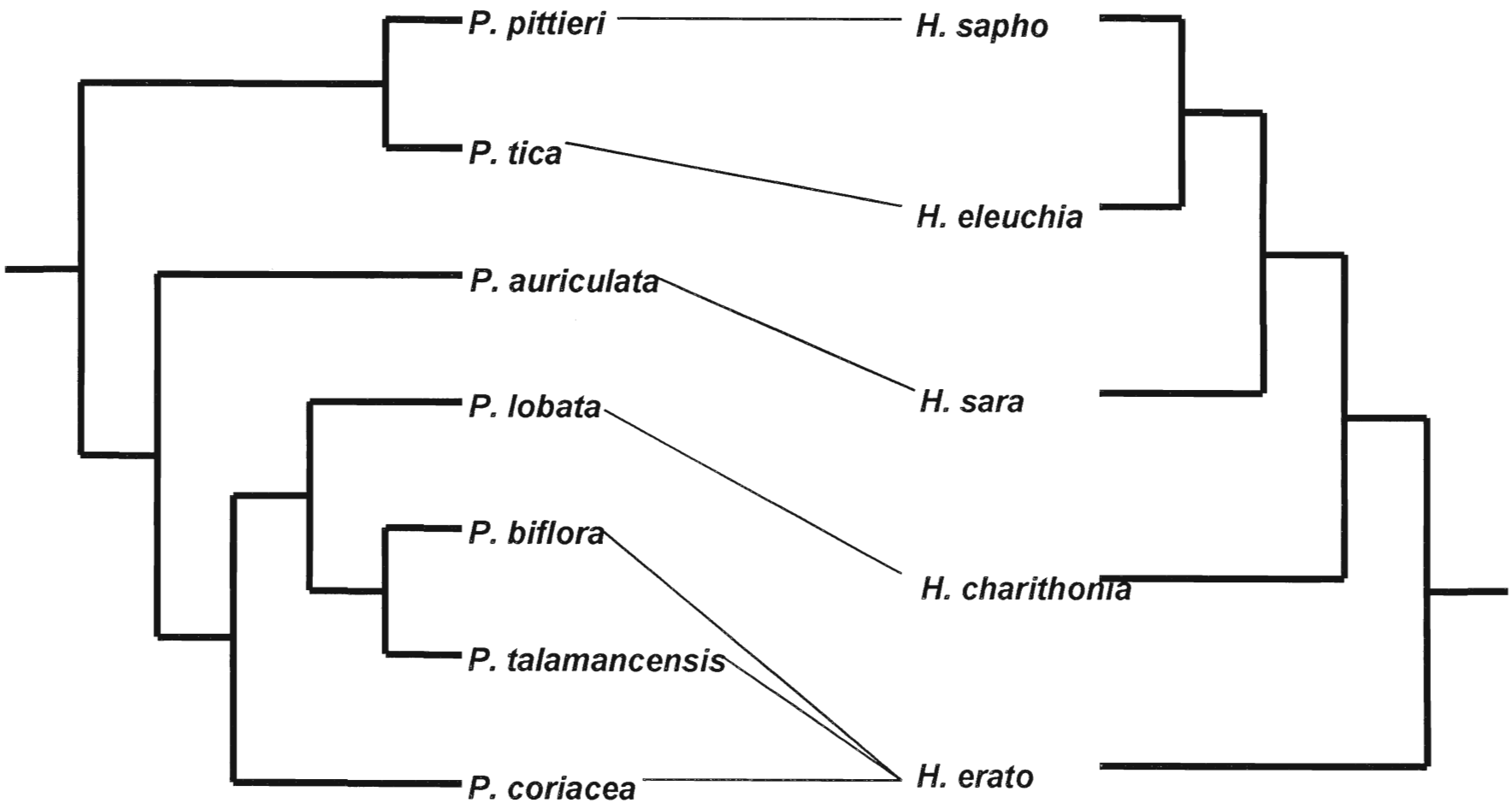

Figure 21. *Decaloba* (*Plectostemma*) - *Astrophea* Feeding Group by topological comparison

Table 15. Matrix listing Passifloraceae hosts and the binary codes for the phylogenetic relationships of the Heliconiinae with hosts listed separately for each parasite usage. (See Appendix H for the phylogeny of this *Passiflora* data set; *Decaloba* phylogeny for this data set is shown in Figure 22)

HOST (Passifloraceae)	PARASITE (Heliconiinae)	BINARY CODE
PASSIFLORA		
P. menispermifolia	4	000101011
P. oerstedii 1	4	000101011
P. oerstedii 2	5	000011011
P. oerstedii 3	2	010000111
P. caerulea	2	010000111
P. platyloba 1	3	001000111
P. platyloba 2	5	000011011
P. ambigua 1	1	100000001
P. ambigua 2	2	010000111
P. quadrangularis	2	010000111
P. alata	3	001000111
**P. vitifolia 1*	2	010000111
**P. vitifolia 2*	5	000011011
DECALOBA		
P. auriculata	3	001000111
P. biflora	1	100000001
P. talamancensis	1	100000001
P. lobata	2	010000011
P. coriacea	1	100000001
**P. pittieri*	5	000011111
**P. tica*	4	000101111

* outgroup(s)

Table 16. Matrix listing Passifloraceae hosts and the binary codes for the phylogenetic relationships of the Heliconiinae with multiple parasites listed per host. (See Figure 22 for the respective phylogenies created from these data sets)

HOST (Passifloraceae)	PARASITE (Heliconiinae)	BINARY CODE
PASSIFLORA		
P. menispermifolia	4	000101011
P. oerstedii	2, 4, 5	010111111
P. caerulea	2	010000111
P. platyloba	3, 5	001011111
P. ambigua	1, 2	110000111
P. quadrangularis	2	010000111
P. alata	3	001000111
**P. vitifolia*	2, 5	010011111
DECALOBA		
P. auriculata	3	001000111
P. biflora	1	100000001
P. talamancensis	1	100000001
P. lobata	2	010000011
P. coriacea	1	100000001
**P. pittieri*	5	000011111
**P. tica*	4	000101111

* outgroup(s)

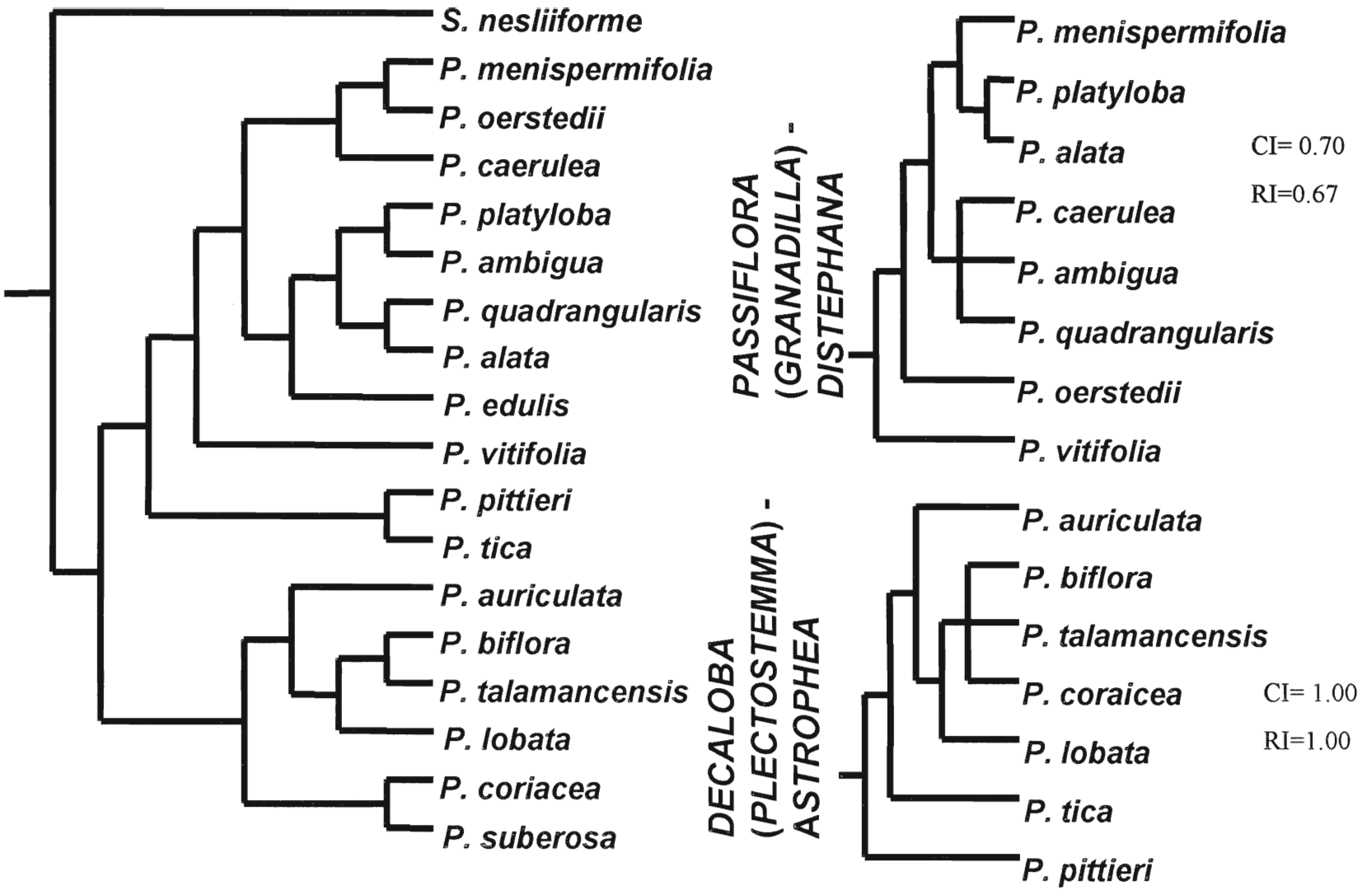

Figure 22. Host-usage Cladogram analysis (Brooks and McLennan, 1991) for *Passiflora* comparing *Decaloba-Astrophea* and *Passiflora - Distephana* groups. The actual *Passiflora* phylogeny based on the combined ITS 1/5.8S/ITS 2 and tRNA-Leucine sequence data is shown at left.

DISCUSSION

IMPLICATIONS OF THE PHYLOGENETIC ANALYSIS FOR HELICONIINAE

The results obtained from the parsimony analysis of the ITS 2 and partial EF-1α sequence data for the 14 species of heliconiines largely support the traditional view of relationships among the heliconiines. The ITS 2 phylogeny (Figure 8) has strong support for the *Heliconius/Laparus* ingroup (bootstrap = 100). This finding supports that of Brown (1981), Brower (1994a), Brower and Egan (1997) as well as the recent morphological representation of Penz (1999). In the EF-1α phylogeny (Figure 9), the same major ingroup clade is also strongly supported (bootstrap = 100) as is this topology in the combined phylogeny (Figure 10).

PRIMITIVE GENERA

Three primitive species were included in this study from three separate genera. These are *Agraulis vanillae* (selected as the outgroup following Brown (1981) and Brower and Egan (1997), *Dryadula phaetusa* and *Dryas iulia*. These were found to be ancestral to the *Heliconius / Laparus* clade in all phylogenetic analyses. In the ITS 2 phylogeny, the relationships for these three genera were slightly different from Brower and Egan (1997). As opposed to sister taxa as in Brower and Egan (1997), *Dryadula phaetusa* was ancestral to *Dryas iulia*. In the EF-1α phylogeny and the combined phylogeny for the heliconiines the relationship amongst the three primitive genera were in agreement with the topology of Brower and Egan (1997), (Figure 1). In the EF-1α tree, (Figure 9) *Dryadula phaetusa* and *Dryas iulia* are supported as sister taxa with a bootstrap value of 96 and in the combined analysis this grouping has a bootstrap value of 58 and a BDI of 2 (Figure 10).

EARLY HELICONIUS RADIATION – LAPARUS DORIS

Laparus doris is a monotypic genus in the Heliconiinae that has been referred to as *Heliconius doris* by some authors (Emsley, 1963; 1965). This species is thought to represent a relic of an early radiation within *Heliconius* although in several previous phylogenies it appears within the *Heliconius* clade (Brower, 1994a; Brower and Egan, 1997; Penz, 1999). In the ITS 2 phylogeny (Figure 8) *L. doris* is supported as ancestral to the 'silvaniform-melpomene' group (bootstrap = 63) which agrees with the placement of this species in Brower and Egan (1997). This placement contrasts with Emsley (1963) and Brown (1981) where *L. doris* is ancestral to the entire *Heliconius* group (Figure 1). In the EF-1α phylogeny and the combined analysis (Figures 9 and 10, respectively) *L. doris* is most closely related to *H. hortense* and ancestral to the 'silvaniform-melpomene' and 'charithonia-sara-sapho' groups. *L. doris* in this topology does not agree with any previous phylogeny. Thus, in the final phylogeny utilized for comparison with the host plant phylogeny the topology was changed to that seen in Figure 17. This figure shows that *L. doris* falls within the *Heliconius* ingroup and shares the common behaviour of pollen feeding which is not seen in the primitive genera. As the ancestor to the 'silvaniform-melpomene' clade, *L. doris* also does not show the behaviourally complex pupal mating behaviour that is considered a more recently evolved trait within the most advanced heliconiines (Benson *et al.*, 1975; Gilbert, 1976; Gilbert, 1991). *L. doris* also shares feeding on the subgenus *Passiflora* in common with the 'silvaniform-melpomene' group giving further support to the placement of this species within this clade.

ADVANCED RADIATION A – THE 'SILVANIFORM-MELPOMERE' GROUP

The placement of the four species of the 'silvaniform-melpomene' group together is very strongly supported in all three phylogenies of the 14 species of Heliconiinae examined (all have bootstrap support of 100). However, the arrangement of the species within the clade does not follow that traditionally accepted by classification or other phylogenetic studies (Emsley, 1963; Benson *et al.*, 1975; Brown, 1981; Brower 1994a; Brower and Egan, 1997; Penz, 1999). As neither of the species arrangements was strongly supported, *H. hecale* and *H. ismenius* were placed as sister taxa and *H. cydno* was placed as a sister species to *H. melpomene* following the traditional arrangements of these species (see Figure 17). As previously mentioned, with *L. doris* this clade represents the non-pupal mating species that feed almost exclusively on passionvines within the *Passiflora* subgenus.

ADVANCED RADIATION B – THE 'ERATO-CHARITHONIA-SARA-SAPHO' GROUP

The remaining five *Heliconius* species fall under the Advanced Radiation B classification of Benson *et al.* (1975) and Brown (1981) (see Figure 17). The grouping of *H. eleuchia*, *H. sapho*, *H. sara* and *H. charithonia* is strongly supported in the ITS 2 and combined phylogenies with bootstrap support values of 99 and 95, respectively. This finding agrees with the traditional view of relationships within this clade as established by Emsley (1963), Brown (1981), Brower (1994a) and Brower and Egan (1997). As the placement of *H. erato* as ancestral to the entire *Heliconius/Laparus* clade was not strongly supported in the three phylogenies established here and because this placement contradicts previously established phylogenies, *H. erato* was placed as ancestral to *H. charithonia* within the 'charithonia-sara-sapho' clade. The consequence of this alteration along with the move of

L. doris to the 'silvaniform-melpomene' group was only a two step increase in overall true length.

The Advanced Radiation B group is considered to be more behaviourally sophisticated amongst the heliconiines. Along with the unique pupal mating behaviour in this group, *H. erato*, *H. charithonia* and *H. sara* utilize *Passiflora* host plants in the subgenus *Decaloba* which are highly inconspicuous in their tropical habitat and thus require behavioural sophistication by host seeking females (Gilbert, 1976; Gilbert, 1991; Smiley, 1985b). Although *H. erato* and *H. charithonia* also exploit pollen sources along with the other *Heliconius* species and *L. doris*, these two species utilize pollen sources other than *Psiguria*. This ability may have afforded a greater success to these two groups in the open habitat where the *Decaloba* are found (Boggs *et al.*, 1981). The larval host plants of the two most derived species in this clade, *H. eleuchia* and *H. sapho*, are in the subgenus *Astrophea* (see Figure 17).

IMPLICATIONS OF THE PHYLOGENETIC ANALYSIS FOR PASSIFLORACEAE

Phylogenetic analyses using parsimony of the 17 species of genus *Passiflora* for each gene region individually and for the two data sets combined resulted in all species being separated along exactly the same lines as the current classification based on morphological description (Killip, 1938; Escobar, 1994; MacDougal, 1994; Vanderplank, 1996). Although not highly resolved, the phylogeny for the tRNA-Leucine data does distribute the taxa into three main subgenera *Passiflora* (with *Distephana*), *Astrophea* and *Decaloba* with strong bootstrap support for each sub grouping (98, 95 and 98, respectively). As no data for the *Astrophea* species (*P. pittieri* and *P. tica*) were available, this subgenus is not represented in

the ITS 1/5.8S/ITS 2 phylogeny (Figure 10). The subgenera *Passiflora* (with *Distephana*) and *Decaloba* are well supported though, as separate clades with bootstrap values of 97 and 100, respectively. The combinations of the two *Passiflora* data sets resulted in the phylogeny seen in Figure 18 which clearly defines all four subgenera.

SUBGENUS: ASTROPHEA

The subgenus *Astrophea* consists of mainly woody bushes and trees that are dispersed throughout the forest canopy with reduced extrafloral nectaries, limited meristems and highly reduced tendrils. This subgenus is thought to be the most primitive group of all *Passiflora* (Killip, 1938; Benson *et al.*, 1975). In the original classification of Passifloraceae, Killip (1938) deems *Astrophea* to be very distinct and almost worthy of generic status. The results of the current study with molecular data support the placement of the two *Astrophea* species, *P. pittieri* and *P. tica*, together (bootstrap=98; BDI=3) and also most ancestral within the *Passiflora/Distephana* clade. This ancestral placement of *Astrophea* is supported by a bootstrap value of 86 and a BDI of 1.

SUBGENUS: DISTEPHANA

The subgenus *Distephana* is represented here by one species: *P. vitifolia*. As a whole, this small yet distinct *Distephana* group consists of highly conspicuous, and brightly coloured woody vines. Morphologically, *P. vitifolia* is a good representative of this subgenus because of its large scarlet flowers and grapevine-like leaves. Due to the lignified stems and the positioning of the flowers' style like the *Astrophea* passionvines, this subgenus is considered to be more ancient than the *Passiflora* (*Granadilla*) subgenus (Benson *et al.*, 1975). Findings here for *Distephana* concur with *P. vitifolia* as ancestral to the *Passiflora*

group (Figure 18). This ancestral placement for *P. vitifolia* is strongly supported with a bootstrap value of 99 and BDI of 4 (Figure 12).

SUBGENUS: PASSIFLORA (GRANADILLA)

This very large and diverse subgenus consists of robust, long-lived vines that grow within the forest edge and is represented by eight species in the current study. Figure 12 shows that all eight of the *Passiflora* species form a clade which is more derived than *Distephana* or *Astrophea*. Morphologically, vines in this subgenus have very large and colourful well-developed flowers which produce equally conspicuous fruit (Killip, 1938; Benson *et al.*, 1975). Extrafloral nectaries are also present in many species of this subgenus and show great variation in structure and location on vegetative parts. Given the diversity and complexity of the *Passiflora* subgenus, the molecular phylogenetic placement of the representatives of this group as more derived than the *Astrophea* or *Distephana* representatives is supported by the morphological classification. The phylogenetic relationships within this subgenus also support the classification to the series level as listed in Table 1 (Killip, 1938; Escobar, 1994; MacDougal, 1994; Vanderplank, 1996). For example, the placement of *P. quadrangularis* with *P. alata* is supported (bootstrap=81; BDI=2) in the final phylogeny for *Passiflora* (Figure 12) and in the classification (Table 1), they both occur under Series 1: Quadrangulares as closely related morphologically.

SUBGENUS: DECALOBA (PLECTOSTEMMA)

The subgenus *Decaloba* is also a large group within the genus *Passiflora* although many of the characteristic features of this group contrast with the subgenus *Passiflora* (*Granadilla*). *Decaloba* passionvines are small, fragile plants with photosynthetic stems that grow close to the ground in open and forest edge habitats (Killip, 1938; Benson *et al.*, 1975;

Vanderplank, 1996). They have small flowers which are often inconspicuous due to their lack of petals or other floral elements. In this group, extrafloral nectaries are only located on leaf surfaces and are often absent entirely. All of these muted features make *Decaloba* vines unapparent in contrast to the showy features of *Passiflora*. *Decaloba* is represented in Figure 12 by six species. Although this subgenus is considered to be the most highly derived of all *Passiflora*, in this molecular phylogeny the *Decaloba* group is strongly supported as the sister clade to *Passiflora*, *Distephana* and *Astrophea* (bootstrap=100; BDI=7). Within this clade there is also strong support for the pairs of sister taxa to section and series level in the classification scheme (Table 1). Firstly, *P. coriacea* and *P. suberosa* are very strongly supported (bootstrap=100; BDI>9) as sister species and occur together in Section 1: Cieca within the classification. Secondly, *P. talamancensis* and *P. biflora* are also very strongly supported as sister groups (bootstrap=100; BDI>9) and these occur together in Section 7: Decaloba; Series 8: Punctatae.

COMPARISON TO OTHER MOLECULAR FINDINGS

The dendogram of genetic relations from Sanchez *et al.* (1999) also finds that *Passiflora* species and numerous accessions (ie. from different geographic locations and samples) group according to classification to the subgenus level. However, the large subgenera *Decaloba* and *Passiflora* are only represented by two and four species respectively with only three of the total of 12 species shared in common with the current study. Previous work by this same group of researchers (Fajardo *et al.*, 1998) failed to separate *Passiflora* or *Decaloba* into distinct groups where 14 species of *Passiflora* and 52 accessions were analyzed (with four species in common with this study). As such, this study represents the first examination of the phylogenetic relationships amongst these 17 species of

Passiflora and, given the agreement with the morphological classification, it represents a strong depiction of the relationships within *Passiflora*. The addition of more sequence data from alternative gene regions and additional species from each subgenus would further elucidate and strengthen the current findings. Also, the addition of sequence data from a more closely related outgroup may prove useful in defining the more ancestral group of the *Passiflora-Distephana-Astrophea* clade and the *Decaloba* clade. The establishment of a morphological phylogeny with which to combine this data set would also further strengthen the hypothesis of cladistic relationships within *Passiflora*.

HELICONIUS / *PASSIFLORA* PHYLOGENY COMPARISON FOR CONGRUENCE

The results from the topological comparison for coevolutionary congruence show that there is not a strict one-to-one relationship of cospeciation between the 14 Costa Rican species of Heliconiinae and their *Passiflora* host plants (Figure 19). However, reciprocal adaptation between groups of phytophagous insects and their host plants occurs very rarely and has not often been examined for antagonistic associations (Farrell and Mitter, 1998; Berenbaum and Passoa, 1999; Clark *et al.*, 2000). As there is a combination of specialist and generalist feeders (Smiley, 1978) amongst the heliconiines, more broad scale congruence for the Heliconiinae and Passifloraceae interaction is expected as opposed to the diffuse coevolution as in Ehrlich and Raven's model (1964). With many polyphagous heliconiines such as *H. cydno*, *H. hecale*, *H. erato* and *H. ismenius*, one-to-one correspondence of insect/plant cannot occur which would translate to a lack of congruence at the species level. However, parallels have been reported for *Heliconius* and *Passiflora* by previous authors at higher taxonomic levels (see Benson *et al.*, 1975).

Due to the lack of congruence in the complete phylogenies for the butterflies and hosts, each group was separated as follows: For the Passifloraceae, the established phylogeny was segregated into phylogenetic relationships of subgenus for each of the major clades formed. In the heliconiine phylogeny, species were separated according to the subgenus of plants on which they feed. The results of these comparisons are shown in Figure 20, the *Passiflora* - *Distephana* feeding group, and Figure 21, the *Decaloba* - *Astrophea* feeding group. In addition, a host cladogram analysis (Brooks and McLennan, 1991) was performed to test for congruence in these two subgroups.

THE PASSIFLORA (GRANADILLA) – DISTEPHANA FEEDING GROUP

The principal radiation of the 'silvaniform-melpomene' group of heliconiines has been onto the *Passiflora – Distephana* subgenus of passionvines (Benson *et al.*, 1975). Innovations that may have facilitated the radiation of these *Heliconius* species onto this robust and varied group of passionvines are believed to have been the initiation of sophisticated behavioural traits. For example, the development of pollen feeding and the seeking and oviposition on meristems by females would likely have been necessary adaptations for this group to succeed on the more advanced *Passiflora – Distephana* passionvines and to coexist with ancestral species (Benson *et al.*, 1975; Gilbert, 1991).

The lack of distinct congruence of host plant use between the 'silvaniform-melpomene' group and the *Passiflora – Distephana* host plants (Figure 20) is due to the polyphagous nature of *H. cydno*, *H. ismenius* and *H. hecale* (Smiley, 1978). According to Benson *et al.* (1975) this lack of patterning or lack of host plant specialization in the butterflies suggests that the radiations responsible for current species occurred relatively recently in evolutionary time. The occurrence of hybrids in these species provides support

for this hypothesis (Benson *et al.*, 1975). The results of the host-usage cladogram analysis (Brooks and McLennan, 1991) for the *Passiflora – Distephana* group also showed minimal congruence between the host-usage cladogram and the original complete phylogeny based on the combined ITS 1/5.8S/ ITS 2 and tRNA-Leucine sequence data (Figure 22).

THE DECALOBA (PLECTOSTEMMA) – ASTROPHEA FEEDING GROUP

The most behaviourally sophisticated group of all the heliconiines is considered to be the 'charithonia-erato-sara-sapho' group which feeds on *Decaloba – Astrophea* passionvines (Benson *et al.*, 1975; Smiley, 1985b). When this *Heliconius* clade (Advanced Radiation B) was compared with the *Decaloba – Astrophea* clade (Figure 21) topologically, there was some congruence. The host searching behaviour in *H. erato* and *H. charithonia* is reportedly highly evolved, a trait which would have enabled the location and occupation of the small and unapparent *Decaloba* meristems on which the females selectively oviposit few or single eggs (Benson *et al.*, 1975). The prevalence of these species in multiple geographic areas is likely a result of this ability to utilize the abundant and widespread yet morphologically cryptic *Decaloba* group (Gilbert, 1975). In addition to this behavioural sophistication, the 'charithonia-erato-sara' group has also evolved a unique pupal mating behaviour (Figure 17) and the ability to utilize non-*Psiguria* pollen sources which has likely also fostered their success.

The most derived of the Advanced Radiation B heliconiines are *H. sapho* and *H. eleuchia*. As Figure 21 shows, these two monophagous species have reverted back onto the ancestral *Astrophea* passionvines. This advanced group exploits the ancestral *Astrophea* species in a novel way by clustering eggs on the meristems which are not used by the

ancestral Heliconiinae which also feed on *Astrophea* (Benson *et al.*, 1975). The host usage cladogram analysis for these groups (Figure 22) yielded a similar amount of congruence as the topological comparison.

HAVE *PASSIFLORA* AND *HELICONIUS* COEVOLVED?

The phylogenetic comparisons of the Heliconiinae with their Passifloraceae host plants showed some coevolutionary congruence, however, strict-sense reciprocal cospeciation has not occurred. The phylogenetic and temporal evidence for these interacting species groups indicate that *Passiflora* and *Heliconius* (particularly the *Decaloba – Astrophea* feeding group) have had a lengthy opportunity to influence each others' evolution through their tight association. The 'arms race' model of coevolution proposed by Ehrlich and Raven (1964) to explain the diversification of plant lineages in response to their corresponding herbivorous butterflies and their reciprocal interactions has yet to be proven convincingly. Moreover, the primary role of plant secondary chemicals in driving this escalation is probably not the main factor in the *Passiflora/Heliconius* association.

In terms of chemical constituents and palatability to the *Heliconius* butterflies, the patterns predicted by Ehrlich and Raven's (1964) 'chemical barrier' process for *Passiflora* are not observed (Smiley, 1978; Smiley 1985b). As the most derived group of passionvines, *Decaloba* would be predicted to have evolved effective chemical barriers to *Heliconius* larval attack resulting in the observed adaptive radiation. As such, only the suitably adapted *Decaloba* feeders should be able to feed on this group and not the *Passiflora –Distephana* feeders. Furthermore, if the *Decaloba* group were derived from ancestors similar to *Passiflora*, then the *Decaloba* feeders should have retained the ability to exploit plants in the

Passiflora subgenus (Smiley 1985b). The results of Smiley (1978) show that this prediction fails for the *Heliconius* butterflies as larval growth rate in *H. erato* is superior only on *Decaloba* host plants while growth in *H. melpomene* and *H. cydno* is more than satisfactory when fed on *Decaloba*, *Distephana* and *Passiflora* host plants (Smiley, 1978).

Therefore, it seems that in *Passiflora* and *Heliconius*, chemical barriers are only one type of defense mechanism and that other traits in both the insect and plant may play a more significant role in influencing the evolution of the association (Benson *et al.*, 1975; Smiley, 1985b; Gilbert, 1991). Specifically in *Passiflora* these traits may include egg mimics, hooked trichomes, cryptic leaf morphology in the *Decaloba* species and indirectly, the presence of extrafloral nectaries utilized by attendant ants (Gilbert, 1971; Gilbert, 1975; Williams and Gilbert, 1981; Smiley, 1985a; Gilbert, 1991). Although these modes of defense are hypothesized to have evolved, in part, due to *Passiflora*'s interaction with Heliconiinae, specific defensive responses between the two groups are somewhat difficult to prove.

The ecological circumstances under which these two associated groups are interacting are vital to understanding their mutual evolutionary history. There are several compelling reasons why parallel cladogenesis may not be seen between insect and host plant phylogenies including (1) there are a number of *Heliconius* species which are polyphagous; (2) host shifts readily occur as seen by the reversion of the 'sapho-eleuchia' pair back onto ancestral passionvines; (3) colonization events may be particularly likely in these Lepidopterans as adults utilize different food resources (i.e. pollen and nectar) than the larval hosts and therefore dispersal and oviposition 'mistakes' are more likely to occur (Berenbaum

and Passou, 1999); and (4) the insects may be host-tracking certain host resources or characteristics (Farrell and Mitter, 1998; Janz and Nylin, 1998).

With the aforementioned degree of ecological interactions occurring between these intimately associated organisms, it is expected that phylogenetic congruence on some level would be observed. Indeed, results here show that, at the subgenus level, parallels can be drawn. However, above this level, strict congruence was not observed which may be due to the relatively recent divergence and radiations for these interacting groups.

Other than topological comparison, statistical means of testing for phylogenetic coevolutionary congruence in groups with complex and multi-factored interactions are limited. As more experimental studies are performed on examples of broad scale coevolution such as the *Heliconius* butterflies and their *Passiflora* host plants, further statistical means of testing such interactions for congruence are necessary.

CONCLUSIONS

This study represents the first phylogenetic comparison of *Heliconius* and *Passiflora* to examine the ecological interactions of association for coevolutionary congruence. Individually, the findings of the *Heliconius* analysis provide additional support for traditional views of the phylogenetic relationships in these Heliconiinae species. For the Passifloraceae, the phylogeny derived from this study strictly follows the morphological classification to subgenus, section and series level. As no phylogeny has yet been published for these host plants, the phylogenetic representation of relationships presented here is a novel addition to *Passiflora* systematics.

The patterns observed by comparing the Passifloraceae and Heliconiinae phylogenetics are suggestive that some parallel evolution in these two groups has occurred. At the subgenus level, there are strong associations between the more evolutionarily advanced clades. A lack of strict congruence beyond the subgenus level, however, suggests that the radiations that produced the current species may have been a recent occurrence in evolutionary time. The multitude of defense and counterdefense characteristics in both *Heliconius* and *Passiflora* that are speculated to be reciprocal ecological adaptations underline the complexity of this coevolutionary interaction. Although plant secondary chemicals are not likely the major factor in influencing coevolution in these two groups as suggested by Ehrlich and Raven (1964), the observed phylogenetic congruence and mutual ecological adaptations indicate that these heliconiines and their host plants have undergone some evolution in parallel.

With the elucidation of additional ecological information on the *Heliconius*/*Passiflora* dynamic and further phylogenetic support for the relationships

amongst the Passifloraceae host plants, a greater understanding of their coevolutionary characteristics will be achieved.

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APPENDICES

APPENDIX A

PCR Reaction Specifications (as specified in the *Taq* PCR Handbook (Qiagen®; October, 1999))

Component	Volume/reaction	Final Concentration
Taq PCR Master Mix	13µl	2.5 units *Taq* DNA Polymerase 200µM each dNTP 1x QIAGEN PCR Buffer
Primers	1µl of each (5µM)	0.1-0.5 µM
Template DNA	Variable*	≤ 1 µg/reaction
Total Volume	**25µl**	

* Template DNA concentration was assessed by estimation on a 1% Agarose gel and by spectrophotometer

APPENDIX B

PCR Protocols

Heliconiinae

Insect ITS 1 / 5.8S / ITS 2 PCR Program

1. Initial Denaturation: 94°C for 3 minutes
2. Cycled Denaturation: 94°C for 1 minute
3. Cycled Annealing: 52°C for 1 minute
4. Cycled Extension: 72°C for 1 minute
5. Go to step 2 for 40 cycles
6. Final Extension: 72°C for 10 minutes
7. Final Refrigeration: 4°C forever

Partial EF-1 alpha PCR Program

1. Initial Denaturation: 94°C for 3 minutes
2. Cycled Denaturation: 94°C for 1 minute
3. Cycled Annealing: 52.5°C for 1 minute
4. Cycled Extension: 72°C for 1 minute
5. Go to step 2 for 35 cycles
6. Final Extension: 72°C for 5 minutes
7. Final Refrigeration: 4°C forever

Passifloraceae

tRNA-Leucine Intron PCR Program

1. Initial Denaturation: 94°C for 3 minutes
2. Cycled Denaturation: 94°C for 1 minute
3. Cycled Annealing: 55.4°C for 1 minute
4. Cycled Extension: 72°C for 1 minute
5. Go to step 2 for 35 cycles
6. Final Extension: 72°C for 5 minutes
7. Final Refrigeration: 4°C forever

ITS 1/ 5.8S / ITS 2 PCR Program

1. Initial Denaturation: 94°C for 3 minutes
2. Cycled Denaturation: 94°C for 1 minute
3. Cycled Annealing: 58.1°C for 1 minute
4. Cycled Extension: 72°C for 1 minute
5. Go to step 2 for 35 cycles
6. Final Extension: 72°C for 5 minutes
7. Final Refrigeration: 4°C forever

APPENDIX C

Heliconiinae ITS 2 Alignment

```
                             10        20        30        40        50        60        70
                    ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Agraulis vanillae   ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
Dryadula phaetusa   ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~ATCTGAGGCCAACGATAAAAAA~CGAGGCAG
Dryas iulia         ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
H. erato            ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
H. cydno            ~~TACTAATATGCTTAAATTCGGCGGGTGATCCTCCCTGATCTGAGGCCAACGATAAAAAAACGAGGCAG
H. melpomene        TNNTAANNTNNTNNNAANTTNNGCGNGTGNTNCTCCCTGATCTGAGGCCAACGATAAAAAAACGAGGCAG
H. hecale           ~~~~~~TNNTANGNNNNNNTNGGNNNGTNNTNCTCNNNGANNNNNNGNNNCCGANANNNAANNGAGGCAG
H. ismenius         ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
H. eleuchia         ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
H. sara             ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
H. sapho            ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
H. charithonia      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
H. hortense         ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~G
L. doris            ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~

                             80        90       100       110       120       130       140
                    ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Agraulis vanillae   ~~~~~~~~~~~~~~~GCGCGAAGGCGCCGATCTCGCCGG~~~~~~~~~GTTAGACGACAACAATAATGTC
Dryadula phaetusa   ACGCGATATCCGTCA.....................A.TTTGGGTCTC~~~~~~~~~~~~~~~~~~~~~~
Dryas iulia         ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~CCA.
H. erato            ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
H. cydno            ACGCGATATCCGNCG........CT..NNNN~....G..GCGGGCGGC...G....CGTT.G.GT.CAG.
H. melpomene        ACGCGATATCCGTCG............NNN......G..GCGGGC~~~...G....CGTT.G..C.CAG.
H. hecale           ACGCGATATCCGTCG.....................G..GCGGGC~~~...G....CGTT.G..T.CAG.
H. ismenius         ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
H. eleuchia         ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
H. sara             ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
H. sapho            ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
H. charithonia      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
H. hortense         ACGCGATATCCGTCA........C~~GGT.......G.NNN~~~~~~~...G....CGTT.G.GT.CCG.
L. doris            ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~

                            150       160       170       180       190       200       210
                    ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Agraulis vanillae   GCGCC~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~ACCCCCGGGCGAGCGTCGGCGTC~GACGCG
Dryadula phaetusa   ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~C..G..C................~......
Dryas iulia         A.CAACAAGTGACGCGGAATCCGCACGCGCGTTACCGCNCGN.A..C.A....N.....N...N......
H. erato            ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
H. cydno            .ACGATCCGCCGCCGTATCTCTCACACTTCCGCCACCCTCCA..~~~.A..............~......
H. melpomene        .ACGATCCGCCGCCGTATCTC~~ACACTTCCGCCACCCTCCT....C.A..............~......
H. hecale           .ACGATCCGCCGCCGTATCTC~~ACACTTCCGCCACCCTCCT....C.A...A..........~......
H. ismenius         ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~ACCTACT....C.A..............~......
H. eleuchia         ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
H. sara             ~~~~~~~~~~~~~~GTCGCTGCACATCTCACACGTCCGCCGTT...CT...............~......
H. sapho            ~~~~~~~~~~~~~~~~~~~~GCACATCTCACACGTCCGCCGTT...CT...............~......
H. charithonia      ~~~~~~~~~~~~~~~~~~~~~~GCACATCTCGCACGTCCGC.....CT.............C.~......
H. hortense         .ACG.GAGTCGGCACATCTC~~~ACACGTCCGCC~~~~~~~.....CT...............~......
L. doris            ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~.....~......

                            220       230       240       250       260       270       280
                    ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Agraulis vanillae   CACTTCGGACGTC~GAGTCCGCCTACTGAGCGCGTACGCAAC~TCTCCAC~~~~~~~~~~~~CGCACAAG
Dryadula phaetusa   .............~..............T..~~.A....T..~..A....CACCACACGTTGTTACGTGT
Dryas iulia         .............N........~....TG..~~......TCAC..A....CA~~~~~~~~~~~~~CTGTT
H. erato            ~~~~~~~~~~~..~.................~~......T.A~~.A.~~~~~~~~~~~~~~~~~~GT..C
H. cydno            .............~.........................T..~A.A.~TATACTCGCTCTA~~~~~~~~~
H. melpomene        .............~.................~~......T..~C.A.~TATACTCGCGCTATA~~~~~~~
H. hecale           .............~.................~~......T..~C.A..TATACTCGCGATATA~~~~~~~
H. ismenius         .............~.................~~......T..~C.A.~TATACTCGCTATA~~~~~~~~~
H. eleuchia         ~~~~~~~~~....~.................~~......T.AC..A.~T.TACTCGCATTGA~~~~~~~~
H. sara             .....T.......~.................~~......T.AC..A.TCTCTCAACT~~~~~....TTGA
H. sapho            .............~.................~~......T.AC..A.~T.TACT~~~~~~~~....TTGA
H. charithonia      .............~.................~~......T.AC..A.TCTTTTGTACTCGCG....TTG~
H. hortense         .............~.................~~......T.AC..A.A~~~~~~~~~~~~~~~~~~~~~~
L. doris            .............~.................~~......T.~C..A.TCTCTCTCTGCT~~~...TGT.~
```

```
                        290       300       310       320       330       340       350
                        ....|....|....|....|....|....|....|....|....|....|....|....|....|
Agraulis vanillae       TG~~CGAAGCGAA~~~CGCGCGCGCACGGCACACAAAG~C~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
Dryadula phaetusa       GACG...GT.CGCGCA....AA..~~.AA....AC..A~.ACAACACGCGTCAACACGCCGAGACGTGTG
Dryas iulia             AA~~..TGA..~~~~~~~..A...~~.ATTG.TT....~.AAC~~~~~~~~~~~~~~~~~~~~~~~~~~
H. erato                A~~~...GCGTG~~~~~~......TG~AA.G.C~....A.ACATCTCTCTACTACGTAGGATATCT~~~~
H. cydno                ~~CG...GA..~~~~~....AN..TG~AA.G...GG..A.ATCTCTATCTCT~~~~~~~~~~~~~~~~~
H. melpomene            ~~CG...GA..~~~~~....A...TG~AA.G...GG..A.ATCTCTATCTCGC~~~~~~~~~~~~~~~~
H. hecale               ~~CG...GA..~~~~~....A...TG~AA.G...GG..A.ATCTCTATCTCT~~~~~~~~~~~~~~~~~
H. ismenius             ~~CG...GA..~~~~~....A...TG~AA.G...GG..A.ATCTCTATCTCT~~~~~~~~~~~~~~~~~
H. eleuchia             ~~~G...GA~~~~~~~....A...TG.AA.G..~....A.ATCTAGAGTATAGACATCA~~~~~~~~~~
H. sara                 G~~~...GA~~~~~~~....A...TG.AA.G..~....A.ACCTAGATTATAGACATCTTTG~~~~~~~~
H. sapho                G~~~...GA~~~~~~~....A...TG.AA.G..~....ATATCTAGAGTATAGACATCAGTG~~~~~~~~
H. charithonia          ~~CG...GTTC.~~~~....A...TG~AA.G..~....A.GTCTAGAGCATAGACATCTGT~~~~~~~~~
H. hortense             ~~~~~~~~~~~~~~~~....A...TG.~A.G..~....A.ATCTACTAGGATATCTGT~~~~~~~~~~~
L. doris                ~~CG...GAGACG~~~....A...TG~AA.G..~~...A.ATCTCTATCT~~~~~~~~~~~~~~~~~~~

                        360       370       380       390       400       410       420
                        ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Agraulis vanillae       ~~~~~~~~~~~~~~~~~~~~~~~GTGCCGAGCGAGCGCACCGGTGTCGGCGC~~CGAGCCAGCTCAACTCC
Dryadula phaetusa       TGCGTGAGTGTGTGCCGTGTGT.C.A..C...C.................~~GA...TC.T..C..CG.
Dryas iulia             ~~~~A~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~.A...~~TA..GG..TA.....
H. erato                ~~~~~~~~~~~~~~~GTGCGTTTGCG..C...G..A.....NN.....~~G..T.G..T.....A..
H. cydno                ~~~~~~~~~~~~~~~~~~~~~~~~~...~~~C...CA~~.....AT.....~~G.CA.T..T.......
H. melpomene            ~~~~~~~~~~~~~~~~~~~~~~~~~...~~~C...CA~~.....AT.....~~G.CA.T..T.......
H. hecale               ~~~~~~~~~~~~~~~~~~~~~~~~~...~~~C...CA~~.....AT.....~~G.CA.T..T.......
H. ismenius             ~~~~~~~~~~~~~~~~~~~~~~~~~...~~~C...CA~~.....AT.....~~G.CA.T..T.......
H. eleuchia             ~~~~~~~~~~~~~~~~~~~~~~~~~.....C...CA~~.....A.....~~T..T.G..T...T.A..
H. sara                 ~~~~~~~~~~~~~~~~~~~~~~~~A..~~~C...CA~~.....A.....GCT..T.G..T...T.AA.
H. sapho                ~~~~~~~~~~~~~~~~~~~~~~~~C..~~~C...CA~~.....A.....~~T..T.G..T...T.A..
H. charithonia          ~~~~~~~~~~~~~~~~~~~~~~~~...~~~C...CA~~.....A.....~~G..T.G..T.....AT.
H. hortense             ~~~~~~~~~~~~~~~~~~~~~~~~...~~~C...CA~~.....A....T..~~G..T.G..T.....A..
L. doris                ~~~~~~~~~~~~~~~~~~~~~~~~...~~~C...CA~~.....AA.....~~G..T.G.....A..

                        430       440       450       460       470       480       490
                        ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Agraulis vanillae       C~GTGGACCCTCTGATTGTTAT~~CTCGAAGGGT~AC~AGGAGAGAGCGGCCCGCTCCTCCCCCGCCGG~
Dryadula phaetusa       .~TC.A.............~~.........T..G.....A....T...G.....A...~
Dryas iulia             .~.......G....GA.T.C.AAT.........~..~.....CA...AA..G.....T
H. erato                .C~.................~~...T.....~G.~.CA.....A...GT...G.....T...T...~
H. cydno                .~.............T....~~.........~..~.....A...GT...G.....T...T...~
H. melpomene            .~.............T....~~.........~..~.....A...GT...G.....T...T...C
H. hecale               .~.............T....~~.........~..~.....A...GT...G.....T...T...C
H. ismenius             .~.............T....~~.........~..~.....A...GT...G.....T...T...~
H. eleuchia             .C.............~~.........~..~.....A...GT...G.....T...T...~
H. sara                 .C.............~~.........~..~.....A...AT...G.....T...T...~
H. sapho                .C.............~~.........~..~.....A...GT...G~....T...T...~
H. charithonia          .C.............~~.........~..~.....A...GT...G.....T...T...~
H. hortense             .~.............~~.........~..~.....A...GT...G.....T...T...~
L. doris                .~............G.....~~.........~..~.....A...GT...G.....T...T...~

                        500       510       520       530       540       550       560
                        ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Agraulis vanillae       AACGCGCGCG~~TCTCGCC~~GCC~~GGATTAGCGGCGG~~CTCGACGGCGC~~~ATTGCCGTTCGCAAT
Dryadula phaetusa       ......T.TCTCAACGC.GCGC~~~~~~~G.~.....~~.GA.....~~G~.....TA
Dryas iulia             T.T....A..CC....TATCCA..GC.CG..G..A...TGT.GA.....AGC..CA.....TA
H. erato                ..........CG....A..C~A..~~..G.G.TA.C...~~.GA...T....GCG~..A...C.....TA
H. cydno                ......A.TACC..CGC.TTA...GTA.CGGCTGC.GC.G~.GA...T....~~~.....CA
H. melpomene            ..T.A.A.GTGTA.~TCG.~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~CA
H. hecale               ..T...A.G~~TA.~TCG.~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~CA
H. ismenius             ......A.~~~TA.CTCG.~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~CA
H. eleuchia             .......TTCCC~~~~A..CCA..~~..G.......G..G~.GA...T....~~~...A.....CA
H. sara                 .......TTCCC~~~~A..C~A..~~..G.........G~.GA...T....~~~...A.....CG
H. sapho                .......TTCCC~~~~A..CCA..~~..G.......G..G~.GA...T....~~~...A.....CA
H. charithonia          ........TCCC~~~~A..C~A..~~..GC.....G~~GA..NT....~~~...A.....CA
H. hortense             ......A.TCCC~~~~A..C~A..~~.............~~.GA...T....~~~...A.....TA
L. doris                ........T.CC~~~~A..~~...~~.T...........~~.GT...A....~~G~.....CG
```

```
                    570        580        590        600        610        620        630
                    ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Agraulis vanillae   GGTGAGT~CGCGATGCGACAGAGGCGTCGACGGCGGCGTTCGCGCGCTCCACACTCACCGTCTCCAGTGC
Dryadula phaetusa   ~......~.....C.....TACC~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
Dryas iulia         ~......C.....C.....TT.CC~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
H. erato            A...T..GT....AA....G.T.CGCCG.C..AGT.T.CCAC.A.A.CGAC~~~~~~~~~~~~~~~~~~~
H. cydno            A...T..~T....A....GTCG.CGCCG.C.AC.~~G.AGGCG....GG.G.~~~~~~~~~~~~~~~~~~~
H. melpomene        A...T..~T....A.NN.GTCG.CGCCG.C.AC...G.AGGCG....GG.G.~~~~~~~~~~~~~~~~~~~
H. hecale           A...T..~T....A....GTC..CGCCG.C.AC...G.AGGCG....GG.G.~~~~~~~~~~~~~~~~~~~
H. ismenius         A...T..~T....A....GTCG.CGCCG.C.AC...G.AGGCG....GG.G.~~~~~~~~~~~~~~~~~~~
H. eleuchia         A...T..~T....A.NNNNG.T.CGC~G.C.~~~AAGAAGGCG...NN.GT.G.G.G.TC....TCTCCG
H. sara             A...T..~T....A.....G.T.CGC~G.C.~~~.AG.AGGCG...GCG.T.G.G.G.TC.A~~TCTACG
H. sapho            A...T..~T....AN....G.T.CGC~G.C.~~~.AG.AGGCG...GC.GT.G.G.G.TC....TCTCCG
H. charithonia      A...T..~T....A.A...G.T.CGC~G.C.~~~~AA.AG~CG...GCG.T.G.G.G.TC..~~TCTCCG
H. hortense         A...T..~T....A.....G.T.CGC~G.C.~~~AAG.AGGCG..CG~~~T.G.G.~~~~~~~.TCTCCG
L. doris            A...T..~T....A.....G.T.C~~~G.C.AC~~~G.AGGCG...GCG.TA.AG.GGG~~~~.TCTCCG

                    640        650        660        670        680        690        700
                    ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Agraulis vanillae   GTGTGTGGAGACGCGCTCGCACCACCGTCGGCC~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
Dryadula phaetusa   ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
Dryas iulia         ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
H. erato            ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
H. cydno            ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
H. melpomene        ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
H. hecale           ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
H. ismenius         ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
H. eleuchia         AGCATCACT.T.T.C~~A.TTTTG.TCGTT.GATAACGCGCTCGCACCGCCCACACCACC~GTAGGCCAC
H. sara             AGCATCACT.T.T.C~~A.TTTTG.TCGTT.GACAACGCGCTCGCACCGCCCACC~~ACC~GTAGGCCAC
H. sapho            AGCATCACT.T.T.C~~A.TTTTG.TCGTT.GATAACGCGCTCGCACCGCCCACC~~ACC~GTAGGCCAC
H. charithonia      AACA.CANT.T.TNCACA.TTT.G.TCGTT~GATAACGCGCTCGCACCACCCACC~~ACN~GTNGGCCAC
H. hortense         AGCA.CACT.T.T.C~~A.TTTTG.TCGGT~GAGAAC~~GCTCGCGCCACCTACC~~ACCGGTAGGCCAC
L. doris            AGC~~~ACT.T.T.C~~A.TT~~G.TCGGTCG.TCGCGG~~TCGCGCCACCTGCC~~GCC~GTCGGCCAC

                    710        720        730        740        750        760        770
                    ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Agraulis vanillae   ~~~ACC~~ATTT~~GATTTGATCGTTATATTTGAGCGGACCGCGGGCGCGAGCTAGGT~CGCC~~~~CCC
Dryadula phaetusa   ~~~~~~~~...C~~.G..~.G..~.....................A...G.C...GT.A~~~~CGCG.T.
Dryas iulia         ~~~~~~~~....CT.G..~.G.............A.........A..~~....GCT.AC...GCGAG...
H. erato            ~~~~~~~~....G~.G..~.G..AC~....A......CG..C..ATA.....A.GT.~~...TCGCA.T.
H. cydno            ~~~..~GAC...~~.G..~.G...C~.........A.........ATA~......GT.~~...TCGCA.T.
H. melpomene        ~~~..~GACA..~~.G..~.G...C~.........A.........ATA~......GT.~~...TCGCA.T.
H. hecale           ~~~..~GACA..~~....~.G...C~.........A.........ATA.......GT.~~...TCGCA.T.
H. ismenius         ~~~..~GACA..~~.G..~.G...C~.........A.........ATA.......GT.~~...TCGCA.T.
H. eleuchia         CAC...GACA..~~.G..~.G..AC~..................ATAC
H. sara             CAC...GACA..~~.G..~.G..AC~..................ATA.......GT.~~...TCGCA.T.
H. sapho            CAC...GACA..~~.G..~.G..AC~..................ATA~......GT.~~...TCGCA.T.
H. charithonia      ACC...CACA..~~.T..~.G..AC~..................ATA....T..GT.G~...TCGCA.T.
H. hortense         CAC...GACA..~~.G..~.G..AC~..................ATA~......GT.~~...TCGCA.T.
L. doris            C~~...GACA..~~.G..~.G..AC~..................ATA.......GT.~~...TCGCA.T.

                    780        790        800        810        820        830        840
                    ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Agraulis vanillae   GACGGAACCGCCATG~TGTACACAACACCGCGTGGACAACAACACCCCAACTCTGCACCGGAGCGAAGAG
Dryadula phaetusa   ..............T~....~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
Dryas iulia         ..........T...CA....~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
H. erato            T...........C.T~...G...GCGCGAA..GC.CGGCGC..TGATGCG~~~~~~~~~~~~~~~~~~~
H. cydno            ..............T~....~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
H. melpomene        ..............T~....~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
H. hecale           ..............T~....~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
H. ismenius         ..............T~....~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
H. eleuchia
H. sara             ..............T~.....G.GCG.A..GCGC.G.GCAGCG.TG.GCG.G~~~~~~~~~~~~~~~~~
H. sapho            ..
H. charithonia      .........C....T~.....G.GCGG.AA..GC.CGGCAC.GTTATGCG.G.GCGCG~~~~~~~~~~~~
H. hortense         ..............T~.....G.G.ACG....GC.CAGTG.TGN.G.GC.
L. doris            ..............A~.....G.GGGCGGCG.C.C.
```

```
                          850       860       870       880       890       900       910
                    ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Agraulis vanillae   TCGTCGCGTCGTCGTCGTCGCAGCGTCGAGCGTACAGTGTGGCTATTGTTTTTATGCAGCCGGCCCTCAG
Dryadula phaetusa   ~~~~~~~~~~~~~~~~~~~.GCGCGGAC.................G.....................
Dryas iulia         ~~~~~~~~~~~~~CG..CGATT.GTG..C...C.............G.....................
H. erato            ~~~~~~~~~~~~~CG..CGCAGTGTCGTC..............A.......................
H. cydno            ~~~~~~~~~~~~~~~~~~~~~C...GTC...............AT......................
H. melpomene        ~~~~~~~~~~~~~~~~~~~~~C...GTC...............AT......................
H. hecale           ~~~~~~~~~~~~~~~~~~~~~C...GTC...............AT......................
H. ismenius         ~~~~~~~~~~~~~~~~~~~~~C...GT
H. eleuchia
H. sara             ~~~~~~~~~~~~~~~~~~~~~C..TGTCG..............A.......................
H. sapho
H. charithonia      ~~~~~~~~~~~~~~~~~~~~~C..T.TCG..............A.......................
H. hortense
L. doris

                          920       930       940       950       960       970       980
                    ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Agraulis vanillae   ACAGGAGTGGTCCTGGATGTAACCCCACGGACCGCAATGTGCGTTCGCAATGTC~ATGTTCTCT
Dryadula phaetusa   ..........................................................A......G......AAATGTGNN
Dryas iulia         ............~~~~~~~~~~~~~~~~~~~~~~
H. erato            ..........................................................A......G......AAATGTTAC
H. cydno            ..........................................................A......G......AAATGTGTG
H. melpomene        ..........................................................A......G......AAATGTGTC
H. hecale           ..........................................................A......G......AAATGTGTC
H. ismenius
H. eleuchia
H. sara             ..........................................................A......G......AAATGTATC
H. sapho
H. charithonia      ..........................................................A......G......AAATGTGGC
H. hortense
L. doris

                          990       1000      1010
                    ....|....|....|....|....|....|....|....
Agraulis vanillae
Dryadula phaetusa   CTGCNNNTCACACTATGACGCGCAGTT
Dryas iulia
H. erato            TCACAA~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
H. cydno            CTGCANNNCACACTATGAC
H. melpomene        CTGC
H. hecale           CTGCA
H. ismenius
H. eleuchia
H. sara             CTGCACT
H. sapho
H. charithonia      CGCAGA
H. hortense
L. doris
```

APPENDIX D

Heliconiinae Partial EF-1α Alignment

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                          10        20        30        40        50        60        70
                  ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Agraulis vanillae ~~GTATTGGTACAGTGCCAGTAGGCAGAGTCGAAACTGGTGTTCTGAAACCCGGTACCATTGTTGTCTTT
Dryadula phaetusa ~T.........................T..........C.................C.....T..C
Dryas iulia       ~~~GTA.T.A..................T..........C.....................C..G..C
H. charithonia    ~TTGT.......................T..........C........T..................
L. doris          ~~~~~~~~~~~~~~G.............T..........C........T..............G...
H. eleuchia       ~~~~~~~~~~~~~~..............T..........CT.......T..................C
H. erato          ~~~~~A......................T..........C........T..................C
H. hecale         ~~~~G.......................T..........C..A.....................G..C
H. ismenius       ~~~~~~~.....................T..........C.......................G..C
H. melpomene      ~~~~~~~.....................T..........C.......................G..C
H. sapho          ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~.....CT.......T..................C
H. cydno          ~~.GTA....~.................T..........C.......................G..C
H. hortense       ~G..........................T..........C........T..................
H. sara           TG..........................T..........CT.......T..................C

                          80        90       100       110       120       130       140
                  ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Agraulis vanillae GCTCCTGCTAACATCACTACTGAAGTTAAGTCCGTTGAAATGCACCACGAAGCTCTCCAAGAGGCTGTGC
Dryadula phaetusa .....C..C........C.....................................................
Dryas iulia       ........C.....................................................
H. charithonia    ..........................A.....................................
L. doris          ........C.................A.....T..........T.....................
H. eleuchia       ..........................A.....................................
H. erato          ..........................A.....T...............................
H. hecale         ..........................A.....................................
H. ismenius       ..........................A.....................................G.....
H. melpomene      ..........................A.....................................
H. sapho          ..........................A.....................................
H. cydno          ..........................A.....T...............................
H. hortense       ..........................A..A..................................
H. sara           ..C.......................A.....................................

                         150       160       170       180       190       200       210
                  ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Agraulis vanillae CTGGAGACAACGTAGGATTCAACGTAAAGAACGTATCTGTCAAGGAATTGCGTCGTGGTTACGTCGCCGG
Dryadula phaetusa .C........T..T..T.....................................................
Dryas iulia       .C........T.....T.....................................................
H. charithonia    ..........T.....T.................G..C.....................................
L. doris          .C........T.....T.................G..C.............................T..
H. eleuchia       ..........T.....T.................G..C.....................................
H. erato          .C........T.....T.................G..C.....................................
H. hecale         .C........T.....T.................G..C.........C...........................
H. ismenius       .C........T.....T.................G..C.........C...........................
H. melpomene      .C........T.....T.................G..C..T......C...........................
H. sapho          ..........T.....T.................G..C.....................................
H. cydno          .C........T.....T.................G..~.........C...........................
H. hortense       .C........T.....T...............T..G..C.....................................
H. sara           ..........T.....T.................G..C.....................................

                         220       230       240       250       260       270       280
                  ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Agraulis vanillae TGACTCTAAAAACAACCCACCCAAGGGAGCCGCTGACTTCACTGCACAAGTCATTGTACTCAACCACCCT
Dryadula phaetusa ......C.....................................C.....................
Dryas iulia       ......C.....................................C.....................
H. charithonia    .........G.....................................C.....................C
L. doris          .........G.............G...T.............C........A.....................
H. eleuchia       ......C..G.................T.............C.....................C
H. erato          ......C..G.................T.....................................
H. hecale         ......C..G...........T.....T.............C.....................C
H. ismenius       ......C..G...........T.....T..T..........C.....................C
H. melpomene      ......C..G...........T.....T.............C.....................C
H. sapho          ......C..G.................T..T..........C.....................C
H. cydno          ......C..G...........T.....T..T..........C.....................C
H. hortense       ......C..G.............G...T.............C.....................
H. sara           ......C..G.................T.............C.....................
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                        290        300        310        320        330        340        350
                    ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Agraulis vanillae   GGTCAAATCTCCAATGGATACACACCTGTGCTGGATTGCCACACAGCTCACATTGCCTGCAAGTTCGCTG
Dryadula phaetusa   ..........T.................T............T.................A.......
Dryas iulia         ............C.............T.................................A.....C.
H. charithonia      ..........T.................T.....................................C.
L. doris            ..............................T.........................A..........C.
H. eleuchia         ..............................T.....................................
H. erato            ..........T.................T.........................T.............
H. hecale           ................C........C...T.............................T.....T..C.
H. ismenius         ................G........C...T.............................T.....T..C.
H. melpomene        ................C........C...T.............................T.....T..C.
H. sapho            ..............................T.....................................
H. cydno            ................C........C...T.............................T.....T..C.
H. hortense         ..........T.................T.....................................C.
H. sara             ..........T.................T.....................................C.

                        360        370        380        390        400        410        420
                    ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Agraulis vanillae   AAATCAAAGAAAAGGTTGACCGTCGTTCTGGTAAATCCACTGAAGAAAATCCCAAATCAATTAAATCTGG
Dryadula phaetusa   ..........................................G........T.....T..C........
Dryas iulia         ..............G.....................T.....G..G...........T..C........
H. charithonia      ..........................................T.....T.....T..C........
L. doris            ..........................................T.....T.....T..C........
H. eleuchia         ..........................................T..C..T.....T..C........
H. erato            ..........................................T.....T.....T..C........
H. hecale           ............A...............................T.....T.....T..C........
H. ismenius         ............A...............................T.....T.....T..C........
H. melpomene        ............A...............................T.....T.....T..C........
H. sapho            ..........................................T..C..T.....T..C........
H. cydno            ............A..............A.............T.....T.....T..C........
H. hortense         ..........................................T.....T.....T..C........
H. sara             ..........................................T.....T.....T..C........

                        430        440        450        460        470        480        490
                    ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Agraulis vanillae   TGACGCCGCTATTGTCAACCTTCAACCATCCAAGCCCCTATGTGTGGAAGCTTTCCAGGAATTCCCTCCC
Dryadula phaetusa   ......T..C..........C.................C........G...................T
Dryas iulia         ......T..C..C........C..G.............C.....................A..........T
H. charithonia      ...T..T.....C........C...........A.....................................A.
L. doris            ...T..T..C........T..C...........A.....G...........C.....A.............
H. eleuchia         ...T..T.....C........C...........A.....G.....................A.........
H. erato            ...T..T.............C...........A.....G.....................A..........~
H. hecale           ...T..T..C..C.....T..C...........A.....G.....................A........C.~T
H. ismenius         ...T..T..C..C.....T..C...........A.....G.....................A........C..T
H. melpomene        ...T..T..C..C.....T..C...........A.....G.....................A........C..T
H. sapho            ...T..T.....C........C...........A.....G.....................A
H. cydno            ...T..T..C..C.....T..C...........A.....G.....................A........C..T
H. hortense         ...T..T.............C...........A.....G.....................A.........
H. sara             ...T..T.....C........C...........A.....G.....................A.........

                        500        510        520        530
                    ....|....|....|....|....|....|....|....|...
Agraulis vanillae   CTCGGTCGTTTCGCCGTGCGT
Dryadula phaetusa   ..........T.........GAG
Dryas iulia         ..............CGTGCGT
H. charithonia      ..........TTG.CGTGCGTATGAGACAAACTGTCGCTGCA
L. doris            ....................ATA
H. eleuchia         ..........T.........
H. erato            ..........T.........GATAGA
H. hecale           ..........CG.GT.ACAT
H. ismenius         ................
H. melpomene        ....................
H. sapho
H. cydno            ..................GTGACATGAGACAAACCGTGGCTGT
H. hortense         ..........T.........AT
H. sara             ..........T.........G
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APPENDIX E

Passiflora ITS 1/5.8S/ ITS 2 Alignment

```
                                10        20        30        40        50        60
                                ....|....|....|....|....|....|....|....|....|....|....|....|
Sphaerocardamum nesliiforme     ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~TCGATGCCTGTC~CAAAACAGAAC~
P. menispermifolia              ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~T.CTG~~....G......~
P. vitifolia                    ~~~~~~~~~~~~~~~~~~~~~~~~AGGATCATTGT~~...AA..~TG~~....G......~
P. oerstedii                    ~~~~~~~~~GGTGAACCTGCGGAAGGATCATTGT~~...AA..~TG~~....G......~
P. platyloba                    ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~CATTGT~~...AA..~TG~~....G......~
P. quadrangularis               ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~..AA..~TG~~....G......~
P. alata                        ~~~~~~~~~~~~~~~~~~~~~CGGAAGGATCATTGT~~...AA..~TG~~....G......~
P. ambigua                      ~~~~~~~~~~~~~~~~~~~~~~~~~NATCATTNNNNNN~~~~..CTG~~.N.TG......~
P. caerulea                     ~~~~~~~~~GGTGAACCTGCGGAAGGATCATTGT~~...AA..~TG~~....G......~
P. edulis                       ~~~~~~~~~GGTGAACCTGCGGAAGGATCATTGT~~...AA..~TG~~....G......~
P. auriculata                   ~~~~~~~~~~~~~~~~~~~~~~AAGGATCATTGT~~.A.AA..~TG~~....G....TAT
P. biflora                      ~~~~~~~~~~~~~~~~~~~~~~AGGATCATTGT~~...AA..~TG~~....G....TAT
P. suberosa                     ~~~~~~~~~~~~~~~~~~~~~~AGGATCATTGT~~...AA..~TG~~....G....CAC
P. coriacea                     ~~~~~~~~~GGTGAACCTGCGGAAGGATCATTGT~~...AA..~TG~~....G....CAC
P. lobata                       ~~~~~~~~~~~~~~~~~~~~~~~~~TCATTGT~~...AA..~TG~~....G....TAC
P. talamancensis                ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~

                                        70        80        90       100       110       120
                                ....|....|....|....|....|....|....|....|....|....|....|....|
Sphaerocardamum nesliiforme     GACCCGCGAACAAACGATCACCACTCGCGGTGGGCTGGTTTCTTAGCC~~~~~~~~GATC
P. menispermifolia              ..........C~GTTG~.GAAGA.NA...C...GC..GGG~CG..~.CGC~~~~~.GG.
P. vitifolia                    ..........C~GTTG~..AA.ACGA...C...GC..GGG~CG..~.CGC~~~~~TGG.
P. oerstedii                    ..........C~GTTG~.GAAGA..A...C...GGC..GGGCGG.~.TGC~~~~~.GG.
P. platyloba                    ..........C~GTTG~.GAA.A..A...C...GC.TGGG~NAG.N.CGC~~~~~.NA.
P. quadrangularis               ..........C~GTTG~.GAA.A.AA...C...GC..GGG~...A~.CGC~~~~~.GG.
P. alata                        ..........C~GTTG~.GAA.A.AA...C...GC..GGG~.G..~.CGC~~~~~.GG.
P. ambigua                      ..........C~GTTG~.GAA.A..A...C...GC.TGGGGAG..~.CGANCCGCAGG.
P. caerulea                     ..........C~GTTG~.GAAGA..A...C...GC..GGG~A.G.~.TGC~~~~~.GG.
P. edulis                       ..........C~GTTG..GAA.ACAA..AC...GC..GGG~CG..~.CGC~~~~~.GG.
P. auriculata                   ............TGTTG~TGAA.A.AAA~~~~~~~~~~GGG~A.TA~.GTC~~~~~TGG.
P. biflora                      ......T.....TGTCG~T.AA.A.AAAA~~~~~~~~.GGG~..G~~TGTT~~~~~.GG.
P. suberosa                     ............TGTTG~TGAA.A.AAAA~~~~~~~~~GGG~..G~~.ATC~~~~~.GG.
P. coriacea                     ..........T.TGTTG~TGAA.A.AAA~~~~~~~~~.GGG~..G~~.ATC~~~~~.GG.
P. lobata                       ............TGTTG.T.AACAAAA~~~~~~~~~..G.G~..GT~GCCC~~~~~~GG.
P. talamancensis                ~~~~~~~~~~~~~~~~~~.AANAAAA~~~~~~~~~~.GGG~..G~~~TTT~~~~~.GGN

                                      130       140       150       160       170       180
                                ....|....|....|....|....|....|....|....|....|....|....|....|
Sphaerocardamum nesliiforme     CC~TTGCCCGCCCGATCCGTGGCTTCGTGTACGGTCCCGGTCGAGAGCTCTATCTCGGTC
P. menispermifolia              A~~~C.T.~~~~~~~C.~.CTC.C~~~.CCC~CTGGA.~~~~~~~~~~~~~~~~~~~~~
P. vitifolia                    ..~~~.T.~~~~~~~C.~.CTCTC~~~.CCC.~CG.A.~~~~~~~~~~~~~~~~~~~~~
P. oerstedii                    A.~~~.T.~~~~~~~C.~.CTC.C~~~.CCC~~CGGA.~~~~~~~~~~~~~~~~~~~~~
P. platyloba                    A.AC~.T.~~~~~~~..~.CCCTC~~~.CCC.CCGGA.~~~~~~~~~~~~~~~~~~~~~
P. quadrangularis               .~~AA...~~~~~~~C.~.CTTT.~~~.T.T.C.GAA.~~~~~~~~~~~~~~~~~~~~~
P. alata                        .~~AA...~~~~~~~C.~.CTCTC~~~.C.CT~CGGA.~~~~~~~~~~~~~~~~~~~~~
P. ambigua                      A.GCATT....T.TC..~TCTC.CC~~~~~~~CC.GA.~~~~~~~~~~~~~~~~~~~~~
P. caerulea                     ..~~~.T.~~~~~~~C.~.CTC..~~~.CCCT~CGGA.~~~~~~~~~~~~~~~~~~~~~
P. edulis                       ..~~~.T.~~~~~~~C.~.CT~~CTC.CCC~CCGGA.~~~~~~~~~~~~~~~~~~~~~
P. auriculata                   A~~...~~~~..ATGA.~.CTCTCCTA..GGG..G.TG.TGT.GA..GGA.CGG.~..G.
P. biflora                      .~~...TAG~..ATGA.~.CTC.CC.A..GGG..A.A~.TGT.GAT.GGGGTGG.~..A.
P. suberosa                     .~~...~~~~..ACGAT~.CTT..C...TGGG..~.GGT.CAT.A.CGAG.GA~.T..G.
P. coriacea                     .~~...~~~~..ATGAT~.CTT.CC.A.TGGG..~.GGT.CAT.AGCTAGCGA~.T..G.
P. lobata                       ..~~..~~~~..ACGGT~.CTC.CCTA..GGG..~~AG.TG..GAT.GGA.CGG.~..G.
P. talamancensis                ..T...T..~..AATA.~TCCC.CA.A..GGG.CA.A~.TGTATAT.GGGGCNG.~.CA.
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                                 190        200        210        220        230        240
                                 ....|....|....|....|....|....|....|....|....|....|....|....|
Sphaerocardamum nesliiforme      TGGTCGTGCGCGTTGCTTCCGGATATC~~~~~~~~~~~ACAAAACCCC~~GGCACAAAAA
P. menispermifolia               ~~~~~~~~~~~~~~~~~~~~~~~~~~.CGCCAC~ACGA.....~~...G.G.G.C
P. vitifolia                     ~~~~~~~~~~~~~~~~~~~~~~~~~~.CGCCCC~ACGA.....~~...G.G.G.C
P. oerstedii                     ~~~~~~~~~~~~~~~~~~~~~~~~~~.CGCCAC~ACGA.....~~...G.T.G.C
P. platyloba                     ~~~~~~~~~~~~~~~~~~~~~~~~~~.CGTCCCAACGA.....~~...G.G.G.C
P. quadrangularis                ~~~~~~~~~~~~~~~~~~~~~~~~~~CGCCAC~ACGA.....~~...G.G...C
P. alata                         ~~~~~~~~~~~~~~~~~~~~~~~~~~.CGCCAA~ACGA.....~~...G.G.G.T
P. ambigua                       ~~~~~~~~~~~~~~~~~~~~~~~~~~.CGCCCCAACGA.....~~...G.G.G.C
P. caerulea                      ~~~~~~~~~~~~~~~~~~~~~~~~~~.CGCCAC~ACGA.....~~...G.G.G.T
P. edulis                        ~~~~~~~~~~~~~~~~~~~~~~~~~~.CGTCCC~ACGA.....~~...G.G.G.T
P. auriculata                    .TTGTCCCAT.C.CC...GT.CGCCGTCCTCCCAACAA.....~~...G.....T
P. biflora                       .T..TCC~AT.C.CTT.....T.CTGTCCTCCCAACAG.AC.....C~...GT....T
P. suberosa                      .A..TCC~AT.~A.TG..TT.C..TGTGCTCCAAACTA.....G~~...G.....T
P. coriacea                      .T..TCC~AT.C..TG..TT.C..TGTCCTCCAAACTA.....G~~...G.....T
P. lobata                        .T..TCC~AT.C.CT...G..T.C.GTCCTCCCAACAA.....~~...GTG...C
P. talamancensis                 GT..TCC~AT.C..TTG.G..T.GTGTCCTCCCAACAGCAC.C.....C~...GT..T.T

                                 250        260        270        280        290        300
                                 ....|....|....|....|....|....|....|....|....|....|....|....|
Sphaerocardamum nesliiforme      GTGTCAAGGAA~CATGTAATGAAG~~~~~~~~~~~CGGT~CGTCATTCGCCTCC~~CCGG
P. menispermifolia               .C.C.......T...AA.~C...AATAC~AGGGAA...G~..G.CC~~.T~GAGCG....
P. vitifolia                     .C.C.......T~CGAA..C...AAGAC~AGGGAA...G~..G.CC~~.T.GGGTG....
P. oerstedii                     .C.C.......T..GAA.~C...AAGAC~AGGGAT...G~..A.CC~~.T.GAGCG....
P. platyloba                     .C.C.......T~CGAA..C...AATACC~GGGAA~..GA..G.CC~~.T.GGGTG....
P. quadrangularis                .C.C.......~.CAAA..C...AAGAT~AGGGAA...G~..G.CC~~.T.G.GTG....
P. alata                         .C.C.......~..AAA..C...AAGAT~AGGGAA...G~..G.CC~~.T.G.GTG....
P. ambigua                       .C.C.......T~CGAA..C...AATATCGGGGA~~..GA..G.CC~~.T.GGGTG....
P. caerulea                      .C.C.......T~CAAA..C...AAAAC~AGGGAAT..G~..G.CCC~.T.GAGTG....
P. edulis                        .C.C.......T..GAA.~C...AAGAC~AGGGAA...G~..G.CC~~.T.G.GTG....
P. auriculata                    .C.C.....T.T.GGAA.T~...AAGTGAAGGGAGT..G~~TG.CC.~AG.~~GTG....
P. biflora                       .C.C.......T.C.AA.T~...AAGAGAAGGGAA.CAG~~CA.CC.~AG.~~GTG....
P. suberosa                      .C.C.......T~CGCA..CA..AAAGAAGGGGA~T.AG~~CA.C..~TT.~~GTT....
P. coriacea                      .C.C.......~.CGAA..CA..AAGAGAAGGGAAT.AG~~CG.CA.~TG.~~GTT....
P. lobata                        .C.C.......T.GGAA.T...TTAGAGAAGGGAGTTAG~~CG.CCA~TGTGATTT~...
P. talamancensis                 .C.C.....TCT.C.A..T~...AAGAAAAGGAAAGCCG~~CC.CA.~AG.~~GTG....

                                 310        320        330        340        350        360
                                 ....|....|....|....|....|....|....|....|....|....|....|....|
Sphaerocardamum nesliiforme      AGACGGTGTG~AGCGCGGATGCCGAGCTGCGATCTAAAGTC~~~~~~~~~TAAAAT~GAC
P. menispermifolia               .N....AT.TCTNN....~C.G.C~C..T.TCGT.CGGAAA~~~~~~~~~C....CN...
P. vitifolia                     .A....AT.TCTC~....GC.G.C~CT.T.TC.T.C.TAAA~~~~~~~~~C....C~...
P. oerstedii                     ......ATCTCTC~....GC.G.C~C..T.TCGT.CGGAAA~~~~~~~~~C....C~...
P. platyloba                     GA....AT.TCTC~.....C.A.C~CT.T.CCGT.CGGAAA~~~~~~~~~C....C~...
P. quadrangularis                .A....AT.TCTC~....GCCG.C~CT.T.TCGG.TG.AAA~~~~~~~~~CC...C~...
P. alata                         .A....AT.TCTC~....GC.G.C~CT.T.TCAT.CGGAAA~~~~~~~~~C....C~...
P. ambigua                       GA....AT.TCTC~....GC.G.C~CT.T.CCGTCCGGAAA~~~~~~~~~C....C~...
P. caerulea                      ......ATCTCTC~....GC.G.C~CA.T.CCGT.CGGAAA~~~~~~~~~C....C~...
P. edulis                        .A....ATCTCTC~....GC.G.C~CT.T.TCCT.CGGAAA~~~~~~~~~C....C~...
P. auriculata                    GA....AA.TCGTT~G..G..G.C.CT.T.CT~A.TC.AAAA~~~~~~~~CT...C~...
P. biflora                       GA....AA.TCGC~AA..GC.G.TG.T.T.CT.G.TTGAAAA~~~~~~~~CT....~...
P. suberosa                      GA....AAATCGT~.A..G..G.T.CT.T.CT.A.TT.AAAA~~~~~~~~.T...C~...
P. coriacea                      G.....AAATCGT~.A..T..G.T.CT.T.CT.A.CT.AAAA~~~~~~~~.C...C~...
P. lobata                        GA....AA.AC.C~AG..G..G.TGT..CGCC.A.TCGAAAA~~~~~~~~CT...C~...
P. talamancensis                 GA...AAT.TCGT~AA..G..T.TG.TCT.CG.G.TTGAA.AA~~~~~~~~T....~...
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```
                                370        380        390        400        410        420
                                ....|....|....|....|....|....|....|....|....|....|....|....|
Sphaerocardamum nesliiforme     TCTCGGCAACGGATATCTCGGCTCTCGCATCGATGAAGAACGTAG~CGAAATGCGATACT
P. menispermifolia              .T.....................N....NN.....N......N~.N...NNT......
P. vitifolia                    ..........................................N..NT.............
P. oerstedii                    ..........................................~.............
P. platyloba                    ..........................................~.............
P. quadrangularis               ..........................................~.............
P. alata                        ..........................................~.............
P. ambigua                      ..................T.........................~.............
P. caerulea                     ..........................................~.............
P. edulis                       ..........................................~.............
P. auriculata                   ..................T.........................~.............
P. biflora                      ..................T.........................~.............
P. suberosa                     ................T.T......T....T...T......N..A~.C......C.....
P. coriacea                     ..................T.........................~.............
P. lobata                       ..................T.........................~.............
P. talamancensis                ..................T.........................~.............

                                430        440        450        460        470        480
                                ....|....|....|....|....|....|....|....|....|....|....|....|
Sphaerocardamum nesliiforme     TGGTGTGAATTGCAGAATCCCGTGAACCATCGAGTCTTTGAACGCAAGTTGCGCCCC~AA
P. menispermifolia              ..............................................NG...G..
P. vitifolia                    ................................................~G..
P. oerstedii                    ................................................~G..
P. platyloba                    ................................................~G..
P. quadrangularis               ................................................~G..
P. alata                        ................................................~G..
P. ambigua                      ................................................~.C
P. caerulea                     ................................................~G..
P. edulis                       ................................................~G..
P. auriculata                   ..................................T.............~G..
P. biflora                      ..................................T.............~A..
P. suberosa                     ..................T...........TC...T.........C.....~G..
P. coriacea                     ..................................T.............~G..
P. lobata                       ..................................T.............~G..
P. talamancensis                ..................................T.............~A..

                                490        500        510        520        530        540
                                ....|....|....|....|....|....|....|....|....|....|....|....|
Sphaerocardamum nesliiforme     GCCTTCTGGCC~GAGGGCACGTCTGCCTGGGTGTCACAAATCGTCGTCCCCCCTCATCTT
P. menispermifolia              ......G..T.A.........N.............NGC..G..T.C......C~...C.
P. vitifolia                    ......G....~.......................TGC.A.....C......CA...CC
P. oerstedii                    ......G..T.~.........C.............TGC.......C......~~...C.
P. platyloba                    ......G....~.......................TGC.......C......~~...C.
P. quadrangularis               ......G....~.......................TGC.......C.....~~~...C.
P. alata                        ......G....~.......................TGC.......C......~~...C.
P. ambigua                      ......G....~.......................TGC.C.....C.....~~~...C.
P. caerulea                     ......G..T.~.......................TGC.......C...T..C....C.
P. edulis                       ......G....~A......................TGC.......C......C~...C.
P. auriculata                   ......G....~.......................TGT.....T.C...~~~~~...CA
P. biflora                      ......G....~.......................TGT.C...T.C...~~~~~...CA
P. suberosa                     ......G....~.......................TGT.....T.C....~~~~...CA
P. coriacea                     .....TG..T.~.......................TGT..T..T.C...~~~~~...CA
P. lobata                       ......G....~.......................TGT.C..CT.C...~~~~~...CA
P. talamancensis                ......G....~.......................TGT.C...T.C...~~~~~T..CA
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                                  550        560        570        580        590        600
                                  ....|....|....|....|....|....|....|....|....|....|....|....|
Sphaerocardamum nesliiforme       T~TGC~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~GGATTCGGGACGGAAGCTGGTCTCCCG
P. menispermifolia                .CC.ACTCCCCCNN~~AGGGGGAA~~~GNGGGTNCGGGAC..G....NAA.........
P. vitifolia                      .CC.ACTCCCCCC~~GAGGGGGAAGA~GGGGGTACGGGGC..G....GA..........
P. oerstedii                      .CC.ACTCCCCCC~~GAGGGGGAA~~~GGGGGTACGGGAT..G....GAA.........
P. platyloba                      .CC.ACTACCCCC~~GACGGGGAA~~~GGGGATACTGCGC..G....GA.....G.T...
P. quadrangularis                 .CC.ACTCCCCCC~~GAGGGGGAA~~~GGGGGTACGGGGT..G....GA..........
P. alata                          .CC.ACTCCCCCC~~GAGGGGGAA~~~GGGGGTACGGGGC..G....GA..........
P. ambigua                        ACC.ACTTCCCCCGTGAGGGGGGGAA~~GGGGTACGGGGC..G....GA..........
P. caerulea                       .CC.GCTCCCCCC~~GAGGGGGAAAAGGGGGGTACGGGAC..G....GAA.........
P. edulis                         .CC.TCTCCCCC~~~GAGGGGAAA~~~GGGGGTACGGGGC..G....GAT.........
P. auriculata                     AC.C~~TTCCTCCAC~~~~~~~~~~AGGAGATC...AAT...G..T..AA.........
P. biflora                        AC.C.CTTT~~~~~~GTGGGGAGATCGG~~ACTA~~~~~...G.....AA.........
P. suberosa                       AC.C.~TTAC~~~~~ATGGGACATTGG~~~ACTA~~~~~...G.....AA.........
P. coriacea                       AC.C.~TTGC~~~~~GTGGGACATTGG~~~AATA~~~~~...G.....AA.........
P. lobata                         AT.C.CTT~~~~~~~GTGGG~AGACAAG~~AGTA~~~~~...G.....AA....T.....
P. talamancensis                  AC.C.CTAT~~~~~~GTGGGGNGATCGG~~ACGA~~~~~...G.....AA.........

                                  610        620        630        640        650        660
                                  ....|....|....|....|....|....|....|....|....|....|....|....|
Sphaerocardamum nesliiforme       TGTGT~TA~CCGCACGCGGTTGGCCAAAATCC~~GAG~CCAAGGACG~CCGGGAGCGTCC
P. menispermifolia                ..C.N~.C~...NT..........G....A.~~..TTTGTT..~..N...A.....C.A
P. vitifolia                      ..C.C~.C~....T..........G....A.~~...TTGTT..~..G...A..A..C.A
P. oerstedii                      ..C.C~.N~....T..........G....A.~~...TTGTT..~..G..TA.....C.A
P. platyloba                      ..C.C~.C~....T..........G....A.~~T.CTTGTT..~..G...ACG.....A
P. quadrangularis                 ..C.C~..~..A.T..........G....A.~~...TTGTT..~..G...A.....C.A
P. alata                          ..C.C~.C~....T..........G....A.~~...TTGTT..~..G...A.....C.A
P. ambigua                        ..C.C~.C~....T..........G....A.~~...TTGTT..~..GT..A.....C.A
P. caerulea                       ..C.C~.C~....T..........G....A.~~...TTGTT..~..G...A.....C.A
P. edulis                         ..C.C~.C~....T..........G....A.~~...TTGTT..~..G...A.....C.A
P. auriculata                     ..C.C~.C~....T.............TT~~...T.GTT..T~~~~~.A~..T.C.A
P. biflora                        ..C.C~CC~....T.............TT~~...T.GTT..T~~~~~.AC..T.C.A
P. suberosa                       .TC.C~.C~....T.....C.......T.~~T..T.GTT..T~~~~~.AT..T.C.A
P. coriacea                       ..C.C~.C~....TA............T.~~T..TTGTT..T~~~~~.AT..T.C.A
P. lobata                         ..C.C~.C~T...T.............AT~~...T.GTT..T~~~~~.AC..T.C.A
P. talamancensis                  ..C.C~CC~T...T.............TT~~...T.GTT..T~~~~~.AC..T.C.A

                                  670        680        690        700        710        720
                                  ....|....|....|....|....|....|....|....|....|....|....|....|
Sphaerocardamum nesliiforme       CGACATGCGGTGGT~G~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
P. menispermifolia                ..G..A........T.T~CGAANACCTTCGGACACCGCCGTGGGCGAGGCCTTCT~GAGG
P. vitifolia                      ..G..A........T.T~CAAAGACCTTCGAAGAATGCCGTCGGCGAGGCCGTCACGAGG
P. oerstedii                      ..G..A........T.T~CGAAGACCTTCGGACACTGCCGTGGGCGAGGCCGTCGCGAGG
P. platyloba                      ..G..A........T.T~CAAAGACCTTCTGAGATTGCCGATGGTCAGGCCGTCACGAGG
P. quadrangularis                 ..G..A........T.T~CAAAGACCTTCGGAGATTGCCGCTGGCGAGGCCGTAACGAGG
P. alata                          ..G..A........T.T~CAAAGACCTTCGGAGATTGCCGCTGGCGAGGCCGTCACGAGG
P. ambigua                        ..G..A........T.T~C~AAGACCTTCGGAGATTGCCGCTGGCGAGGCCGTCACGAGG
P. caerulea                       ..G..A........T.T~CAAAGACCTTCGGAGATTGCCGTCGGCGAGGCCGTACTAAAA
P. edulis                         ..G.~A...~....T.T~CAAAGACCTTCGGAGATTGCCGCCGGCGAGGCTGTCACGGG~
P. auriculata                     ..G.TA........T.~~ATAAGACCTTCGAAAAATGCCGCGGCCAAG~CCAACAAAAGG
P. biflora                        ..G..AT.......T.~~ATAAAACCTTCGCAAAATGCCGTGGACGAG~CCAACAGAAGG
P. suberosa                       ..G..A........T.~~ATAAAACCTTCGGAAAATGTCGTGACCAAG~CCAACAAAAGA
P. coriacea                       ..G..A........T.~~ATAAAACCTTCGAAAAATGTCGTGACCAAG~CCAACAAAAGA
P. lobata                         ..G..A........T.~~ACAAAACCTTCGAAAAATGCCACGGCCAAG~CCAACAAAAGG
P. talamancensis                  ..G..AT.......T.~~ATAAAACCTTCGCAAAATGTCGTGGACGAG~CCAACAGAAGG
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                                730       740       750       760       770       780
                                ....|....|....|....|....|....|....|....|....|....|....|
Sphaerocardamum nesliiforme     ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
P. menispermifolia              CTCCGGGA~~~~~~~~~~~~CCCTGTTTTNTAACCACANNGACCNCAGGTNAGGCNGGAT
P. vitifolia                    CTCCGGGA~~~~~~~~~~~~CCCTGTTTTCCAACCACGGCGACCCCAGGTCAGGCGGGAT
P. oerstedii                    CTCCGGGA~~~~~~~~~~~~CCCTGTTTTCTAACCACAGCGACCCCAGGTCAGGCGGGAT
P. platyloba                    CTGCTGGA~~~~~~~~~~~~CCCTGTTTTCTACCCACAGCGACCCCAGGTCANGCGGGAT
P. quadrangularis               CTCCGGGA~~~~~~~~~~~~CCCTGTTTTCTCACCACGGCGACCCCAGGTCAGGCGGGAT
P. alata                        CTCCGGGA~~~~~~~~~~~~CCCTGTTTTCTCACCACGGCGACCCCAGGTCAGGCGGGAT
P. ambigua                      CTCCGGGA~~~~~~~~~~~~CCCTGTTTTCTACCCACAGCGACCCCAGGTCAGGCGGGAT
P. caerulea                     TCTCGGGA~~~~~~~~~~~~CCCTGTTCTCTAACCACAGCGACCCCAGGTCAGGCGGGAT
P. edulis                       CTCCGGGA~~~~~~~~~~~~CCCTGTTTTCTAACCACAGCGACCCCAGGTCAGGCGGGAT
P. auriculata                   CTCCGAGA~~~~~~~~~~~~CCCTGCTCACTCCCAACAGCGACCCCAGGTCAGGCGGGAT
P. biflora                      CTCTGAGA~~~~~~~~~~~~CCCTGTTCACTCNCAACAGCGACCCCAGGTCAGGCGGGAT
P. suberosa                     CTATTTGA~~~~~~~~~~~~CCCTGTCCACTCCCAACAGCGACCCCAGGTCAGGCGGGAT
P. coriacea                     CTATTTGA~~~~~~~~~~~~CCCTGTCCACTCCCAATAGCGACCCCAGGTCAGGCGGGAT
P. lobata                       CTTCGAGA~~~~~~~~~~~~CCCTGTTCACTCCCAACAGCGACCCCAGGTCAGGCGGGAT
P. talamancensis                CTTCGAGA~~~~~~~~~~~~CCCTGTTCACTCCCAACAGCGACCCCAGGTCAGGCGGGAT

                                790       800       810       820       830       840
                                ....|....|....|....|....|....|....|....|....|....|....|
Sphaerocardamum nesliiforme     ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~AA~~~~TTC~~~GAT~~CCACT
P. menispermifolia              CACCCGCTNNGTNNAAGCATATCAA~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
P. vitifolia                    TACCCGCTGAGNNNNANCATATCAATAAGCGGAGGAA~~~~~~~~~~~~~~~~~~~~~~~
P. oerstedii                    CACCCGC~GAGTTTAAGCATATCAATAAGCGGAGGAGATGCCAGAG.CATA..AT..GT.
P. platyloba                    CACCCGCTGAGTTTAANNANATCAATAAGCGGAGGAGG.C~~~~~~~~~~~~~~~~~~~~
P. quadrangularis               TACCCGCTGAGTTTAAGCATATCAATAAGCGGAGGAA~~~~~~~~~~~~~~~~~~~~~~~
P. alata                        TACCCGCTGAGTTTAAGCATATCAATAAGCGGNGGAA~~~~~~~~~~~~~~~~~~~~~~~
P. ambigua                      CACCCGCTGAGTTTAAGCATATCAATAAGCGGAGGAAGG.~~~~~~~~~~~~~~~~~~~~
P. caerulea                     ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
P. edulis                       TACCCGATGAG~~~~~~~~~~TCAATAAGCGGAGGGAA~~~~~~~~~~~~~~~~~~~~~~
P. auriculata                   TACCCGCTGAGNNTNAGCATATCAATAAGCGGAGGAA~~~~~~~~~~~~~~~~~~~~~~~
P. biflora                      NACCCGCTNAGNNTAANNAN~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
P. suberosa                     TACCCGNTGAGNTNANCCTTATAATTAACCGGAGGAA~~~~~~~~~~~~~~~~~~~~~~~
P. coriacea                     TACCCGCTGAGTTTAAGCATATCAATAAGCG~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
P. lobata                       TACCCGCTGAGTTTAAGCATATCAATAAGC~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
P. talamancensis                TACCCGCTGAGTTTAAGCATATCAATAAGCGGAGGAA~~~~~~~~~~~~~~~~~~~~~~~

                                850       860       870       880       890       900
                                ....|....|....|....|....|....|....|....|....|....|....|....|
Sphaerocardamum nesliiforme     CTCATAT~CGTC~GGCCGCTCCTGTCTGGAAGCTCTATAGTTGACCCAAAGTCCTCAAAG
P. menispermifolia              ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
P. vitifolia                    ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
P. oerstedii                    GC.GAGAGTCGTTTTGTTTC.AAACGA.A.G.GG.CGGCCAGC.AGAG.TCCGTGTCCG.
P. platyloba                    ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
P. quadrangularis               ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
P. alata                        ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
P. ambigua                      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
P. caerulea                     ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
P. edulis                       ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
P. auriculata                   ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
P. biflora                      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
P. suberosa                     ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
P. coriacea                     ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
P. lobata                       ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
P. talamancensis                ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
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                                       910       920       930       940       950       960
                               ....|....|....|....|....|....|....|....|....|....|....|....|
Sphaerocardamum nesliiforme    CG
P. menispermifolia             ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
P. vitifolia                   ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
P. oerstedii                   .ACTGGACGGGTCACCCGAGCCCTGTCTTTTCGGATCTGATGCCTTGGCGACGCTAACGC
P. platyloba                   ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
P. quadrangularis              ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
P. alata                       ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
P. ambigua                     ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
P. caerulea                    ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
P. edulis                      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
P. auriculata                  ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
P. biflora                     ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
P. suberosa                    ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
P. coriacea                    ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
P. lobata                      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
P. talamancensis               ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~

                                       970       980       990      1000      1010      1020
                               ....|....|....|....|....|....|....|....|....|....|....|....|
Sphaerocardamum nesliiforme
P. menispermifolia             ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
P. vitifolia                   ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
P. oerstedii                   CGGGGTTTTGTTCGTGTGGCCGGTCCGGGAAGGGAACGTGACTTGACCCAAGCCCGCCCC
P. platyloba                   ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
P. quadrangularis              ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
P. alata                       ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
P. ambigua                     ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
P. caerulea                    ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
P. edulis                      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
P. auriculata                  ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
P. biflora                     ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
P. suberosa                    ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
P. coriacea                    ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
P. lobata                      ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
P. talamancensis               ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~

                               ....|.
Sphaerocardamum nesliiforme
P. menispermifolia             ~~~~~~
P. vitifolia                   ~~~~~~
P. oerstedii                   CGCCCG
P. platyloba                   ~~~~~~
P. quadrangularis              ~~~~~~
P. alata                       ~~~~~~
P. ambigua                     ~~~~~~
P. caerulea                    ~~~~~~
P. edulis                      ~~~~~~
P. auriculata                  ~~~~~~
P. biflora                     ~~~~~~
P. suberosa                    ~~~~~~
P. coriacea                    ~~~~~~
P. lobata                      ~~~~~~
P. talamancensis               ~~~~~~
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APPENDIX F

Passiflora tRNA-Leucine Intron Alignment

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                                10        20        30        40        50        60
                                ....|....|....|....|....|....|....|....|....|....|....|....|
Sphaerocardamum nesliiforme     ~~~~~~~~~~~~~~~AATTGGATTGAGCCTTGGTATGGAAACCTACNAAGTGATAACTTT
P. alata                        ~~~~~~~~~~~~CTT.........................T...T.............
P. ambigua                      ~~~~~~~~~~~~GCTT.........................T...T.............
P. auriculata                   ~~~~~~~~~~~~GCTT.........................T...T.............
P. biflora                      ~~~~~~~~~~GNCTT.........................T...T.............
P. coraicea                     ~~~~~~~~GNNNNTT.........................T...T.............
P. edulis                       ~~~~~~~~~~GNCTT.........................T...T.............
P. caerulea                     ~~~~~~~~~~GNCTT.........................T...T.............
P. menispermifolia              ~~~~CTNNNNGNCTT.........................T...T.............
P. oerstedii                    ~~~~~~~~~~GACTT.........................T...T.............
P. lobata                       ~~~~~~~~~~~GNNTTNN.......................T...T.............
P. mollissima                   ~~~~~~~~~~~~TT..........................T...T.............
P. pittieri                     ~~~~~~~~~~~~~~~~~~~.....................T...T........G....
P. platyloba                    ~~~~~~TNNNGNCTT.........................T...T.............
P. quadrangularis               ~~~~~~~~~~~~~~~~~~~.....................T...T.............
P. suberosa                     ~GNNGNTNNNNNCTT.T.N..N.N.................T...T.............
P. talamancensis                ~~~~~~~~~~GNCTT.........................T...T.............
P. vitifolia                    ~~~~~~~~~~~~~~~~~~~.....................T...T.............

                                    70        80        90       100       110       120
                                ....|....|....|....|....|....|....|....|....|....|....|....|
Sphaerocardamum nesliiforme     CAAATTCAGAGAAACCCTGGAATTAACAATGGGCAATCCTGAGCCAAATCCTGGTTTACG
P. alata                        ......................A..A.........................T...T..
P. ambigua                      ......................A..A.........................T...T..
P. auriculata                   ......................C..A.......................TT...TTC
P. biflora                      ......................C..A.......................TT...TTC
P. coraicea                     ......................A..A.......................TT...TGT
P. edulis                       ......................A..A.........................T...T..
P. caerulea                     ......................A..A.........................T...T..
P. menispermifolia              ......................A..A.........................T...T..
P. oerstedii                    ......................A..A.........................T...T..
P. lobata                       ......................C..A.......................TT...TTT
P. mollissima                   ......................A..A.........................T...T..
P. pittieri                     ......................C..A.........................T...T..
P. platyloba                    ......................A..A.........................T...T..
P. quadrangularis               ......................A..A.........................T...T..
P. suberosa                     ......................C..A.......................TT...TTT
P. talamancensis                ......................C..A.......................TT...TTT
P. vitifolia                    ......................A..A.........................T...T..

                                   130       140       150       160       170       180
                                ....|....|....|....|....|....|....|....|....|....|....|....|
Sphaerocardamum nesliiforme     ~~~~~~~~~~~~~CGAACAA~~~~~~~~~~~ACCGGAGTTTAGAAAGCGAGAA~~~~~~~
P. alata                        ~~~~~~~~~~~~~AA.....AAAAA~~~~CA..AAAG...C.T....AC....TCAGAAT
P. ambigua                      ~~~~~~~~~~~~~AA.....AAAAA~~~~CA..AAAG...C.T....AC....TCAGAAT
P. auriculata                   ~~~~~~~~~~~~GAA.....AAAAAAA~~TA..AAAG...C.T....AC....T~~~~~~
P. biflora                      ~~~~~~~~~~~~GAA.....AAAAAAAAATA..AAAG...C.T....AC....T~~~~~~
P. coraicea                     TATT~~~~~~~~GTTTT...AAAAAAA~~GA..AAAG...C.T....AC....T~~~~~~
P. edulis                       ~~~~~~~~~~~~~AA.....AAAAA~~~~CA..AAAG...C.T....AC....TCAGAAT
P. caerulea                     ~~~~~~~~~~~~~AA.....AAAAA~~~~CA..AAAG...C.T....AC....TCAGAAT
P. menispermifolia              ~~~~~~~~~~~~~AA.....AAAAA~~~~CA..AAAG...C.T....AC....TCAGAAT
P. oerstedii                    ~~~~~~~~~~~~~AA.....AAAAA~~~~CA..AAAG...C.T....AC....TCAGAAT
P. lobata                       TTT~~~~~~~~~GAA.....AAAAAAAA~~T...AAG...C.T....AC....T~~~~~~
P. mollissima                   ~~~~~~~~~~~~~AA.....AAAAA~~~~CA..AAAG...C.T....AC....TCAGAAT
P. pittieri                     ~~~~~~~~~~~~~AA.....AAAAA~~~~CA..AAAG...C.T....AC....TCCGAAT
P. platyloba                    ~~~~~~~~~~~~~AA.....AAAAA~~~~CA..AAAG...C.T....AC....TCAGAAT
P. quadrangularis               ~~~~~~~~~~~~~AA.....AAAAA~~~~CA..AAAG...C.T....AC....TCAGAAT
P. suberosa                     TCTTTTTTTTTTGAA..A..AAAAAGAAA~~~~AAAG...C.T....AC....T~~~~~~
P. talamancensis                C~~~~~~~~~~GAA.....AAAAAAAA~TA..AAAG...C.T....AC....T~~~~~~
P. vitifolia                    ~~~~~~~~~~~~~AA.....AAAA~~~~CA..AAAG...C.T....AC....TCAGAAT
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                                 190        200        210        220        230        240
                                 ....|....|....|....|....|....|....|....|....|....|....|....|
Sphaerocardamum nesliiforme      ~AA~AAGGGATAGGTGCAGAGACTCAATGGAAGCTGTTCTAACAAATGGAGTTCACTACC
P. alata                         A..T..A.....................C.........................G.T.G.G
P. ambigua                       A..T..A.....................C.........................G.T.G.G
P. auriculata                    A..~..A.....................C.....T...................G.T.G.G
P. biflora                       A..~..A.....................C.....T...................G.T.G.G
P. coraicea                      A..~..A.....................C.....T...................G.T.G.G
P. edulis                        A..T..A.....................C.........................G.T.G.G
P. caerulea                      A..~..A.....................C.........................G.T.G.G
P. menispermifolia               A..~..A.....................C.........................G.T.G.G
P. oerstedii                     A..~..A.....................C.........................G.T.G.G
P. lobata                        ~..~..A..~.~................C.....T...................G.T.G.G
P. mollissima                    A..~..A.....................C.........................G.T.G.G
P. pittieri                      A..~..A.....................C.........................G.T.G.G
P. platyloba                     A..T..A.....................C.........................G.T.G.G
P. quadrangularis                A..T..A.....................C.........................G.T.G.G
P. suberosa                      A..~..A.....................C.....T...................G.T.G.G
P. talamancensis                 A..~..A.....................C.....T...................G.T.G.G
P. vitifolia                     A..T..A.....................C.........................G.T.G.G

                                 250        260        270        280        290        300
                                 ....|....|....|....|....|....|....|....|....|....|....|....|
Sphaerocardamum nesliiforme      TTGTGTTGATAAAGGAATCCTTCGATCGAAACT~~~~~~~~~~~~~~~~~~~~~~~TCAG
P. alata                         ...C~~~~.............T..TA.....~~~~~~~~~~~~~~~~~~~CCAGAAA..
P. ambigua                       ..AC~~~~.............T..TA.....~~~~~~~~~~~~~~~~~~~CCAGAAA..
P. auriculata                    ....~~~~.....A.......T..TA.....~~~~~~~~~~~~~~~~~~~CCAGAAA..
P. biflora                       ....~~~~C....A.......TT.TA.....~~~~~~~~~~~~~~~~~~~TCAGAAA..
P. coraicea                      ..C.~~~~.....AA......T..TA.....~~~~~~~~~~~~~~~~~~~CCAGAAA..
P. edulis                        ...C~~~~.............T..TA.....~~~~~~~~~~~~~~~~~~~CCAGAAA..
P. caerulea                      ...C~~~~.............T.GTA.....~~~~~~~~~~~~~~~~~~~CCAGAAA..
P. menispermifolia               ...C~~~~.............T.GTA.....~~~~~~~~~~~~~~~~~~~CCAGAAA..
P. oerstedii                     ...C~~~~.............T.GTA.....TAG~~~~~~~~TAAAACTCCAGAAA..
P. lobata                        ....~~~~.....A.......T..TA.....~~~~~~~~~~~~~~~~~~~CCAGAAA..
P. mollissima                    ...C~~~~.............T..TA.....~~~~~~~~~~~~~~~~~~~CCAGAAA..
P. pittieri                      ...C~~~~.............T..TA.T.T.AAAACTAATATTAAAACTCCAGAAA..
P. platyloba                     ...C~~~~.............T..TA.....~~~~~~~~~~~~~~~~~~~CCAGAAA..
P. quadrangularis                ...C~~~~.............T..TA.....~~~~~~~~~~~~~~~~~~~CCAGAAA..
P. suberosa                      ..C.~~~~.....A.......TT..TA.....~~~~~~~~~~~~~~~~~~~CCAGAAA..
P. talamancensis                 ....~~~~.....A.......T..TA.....~~~~~~~~~~~~~~~~~~~TCAGAAA..
P. vitifolia                     ...C~~~~.............T..TA.....~~~~~~~~~~~~~~~~~~~CCAGAAA..

                                 310        320        330        340        350        360
                                 ....|....|....|....|....|....|....|....|....|....|....|....|
Sphaerocardamum nesliiforme      ATGAAG~~~~~~~~~~~~~~~~~GAGA~AAAAC~CTATATTTAGACAATATAGGTAACAC
P. alata                         ..A...~~~~TGATAAAA~~~~TA.A.G.T...A...ATA.ACATAG..CC.C...CTGA
P. ambigua                       ..A...~~~~TGATAAAA~~~~TA.A.G.T...A...ATA.ACATAG..CC.C...CTGA
P. auriculata                    .GA...~~~~TGATAAAA~~~~TA.A.G.T...A...ATA.ACATAG..C..C...CTGA
P. biflora                       ..A...~~~~TGATAAAA~~~~TA.A.G.T..GA.G.ATA.ACATAG..C..C...CTGA
P. coraicea                      ..A...~~~~TGCTAAAA~~~~TA.A.G.T...A...ATA.ACATAG..C..C...CTGA
P. edulis                        ..A...~~~~TGATAAAA~~~~TA.A.G.T...A...ATA.ACATAG..CC.C...CTGA
P. caerulea                      ..A...AAAGTGATAAAA~~~~TA.A.G.T...A...ATA.ACATAG...C.C...CTGA
P. menispermifolia               ..A...~~~~TGATAAAA~~~~TA.A.G.T...A...ATA.ACATAG..CC.C...CTGA
P. oerstedii                     ..A...~~~~TGATAAAA~~~~TA.A.G.T...A...ATA.ACATAG..CC.C...CTGA
P. lobata                        ..A...~~~~TGATAAAA~~~~TA.A.G.T...A...ATA.AGATAG..C..C...CTGA
P. mollissima                    ..A...~~~~TGATAAAA~~~~TA.A.G.T...A...ATA.ACATAG..CC.C...CTGA
P. pittieri                      ..A...~~~~TGATAAAAAAAATA.A.G.T...A...ATA.ACATAG..C..CA..CTGA
P. platyloba                     ..A...~~~~TGCTAAAA~~~~TA.A.G.T...A..CATA.ACATAG..CC.C...CTGA
P. quadrangularis                ..A...~~~~TGATAAAA~~~~TA.A.G.T...A...ATA.ACATAG..CC.C...CTGA
P. suberosa                      ..A...~~~~TGCTAAAA~~~~TA.A.G.T.C.A...ATA.ACATAG..C..C...CTGA
P. talamancensis                 ..A...~~~~TGATAAAA~~~~TA.A.G.T...A.G.ATA.ACATAG..C..C...CTGA
P. vitifolia                     ..A...~~~~TGATAAAA~~~~TA.A.G.T...A...ATA.ACATAG..CCGC...CTGA
```

```
                                370        380        390        400        410        420
                                ....|....|....|....|....|....|....|....|....|....|....|....|
Sphaerocardamum nesliiforme     AAAACGATCTCAAA~~~~~~~~~~~AATGACGA~~~~CCTGAA~~~~~TCTCGATTTCT~
P. alata                        ..T..T........TACAAATAATT........GCGA..CA..~CCTG.A..T..A.T.T
P. ambigua                      ..T..T........TACAAATAATT........GCGA..CA..~CCCG.A..T..A.T.T
P. auriculata                   ..T..T........TACAAATAATT........GCGA...C..~TCTG.A..T..A.T.T
P. biflora                      ..T..T........TACAAATAATT...A...GGCGA..CC..~TCTG.A..T..A.T.T
P. coraicea                     ..T..T..T.....TAGAAATAATT........GCGA..CC..~TCTA.A..T..A.T.T
P. edulis                       ..T..T........TACAAATAATT........GCGA..CA..~CCTG.A..T..A.T.T
P. caerulea                     ..T..T........TACAAATAATT........GTGATACA..~CCTG.A..T..A.T.T
P. menispermifolia              ..T..T........TACAAATAATT........GTGAT.CC..~CCTG.A..T..A.T.T
P. oerstedii                    ..T..T........TACAAATAATT........GTGAT.CA..~CCTG.A..T..A.T.T
P. lobata                       ..T..T...ATT..TACAAATAATT........GCGA..CC..~TCTGGA..T..A.T.T
P. mollissima                   ..T..T........TACAAATAATT........GCGA..CA..~CCTG.A..T..A.T.T
P. pittieri                     ..T..T........TACAAATAATT........GCAA..CA..CTCTG.A..T..A.T.T
P. platyloba                    ..T..T........TACAAATAATT........GCGA..CA..~CCTG.A..T..A.T.T
P. quadrangularis               ..T..T........TACAAATAATT........GCGA..CA..~CCTG.A..T..A.T.T
P. suberosa                     ..T..T........TATAAATAATT........GCGA..CC..~TCTA.A..T..A.T.T
P. talamancensis                ..T..T........TACAAATAATT...A....GCGA..CC..~TCTG.A..T..A.T.T
P. vitifolia                    ..T..T........TACAAATAATT........GCGA..CA..~CCTG.A..T..A.T.T

                                430        440        450        460        470        480
                                ....|....|....|....|....|....|....|....|....|....|....|....|
Sphaerocardamum nesliiforme     ~~~~ATTTTTTTATAA~~~~~A~~~~~~~~CAAAAT~~~~~~CGAAATGGTATGAATAAA
P. alata                        TTCTG.A.C...T.TTTTT~C.TTTTTTT~~.T..A~~~~~~AA.GT..T.C.....C..
P. ambigua                      TCCTG.A.C...T.TTTT~~C.TTTTTTT~~.T..A~~~~~~AA.GTA.T.C.....C..
P. auriculata                   TCTTG.A.C...T.TTTTTTC.TTTTTT~~..T..A~~~~~~AA..GG.T.C.....C..
P. biflora                      TCTTG.A.C...T.TTTTT~C.TTTTTT~~..T..A~~~~~~AA..T..T.C.....C..
P. coraicea                     TCTTG.A.C...T.TTTTTAATTTTTTTTT.~TG.AA~~~~~AA..T..T.C.....CCT
P. edulis                       TCCTG.A.C...T.TTTTT~C.TTTTTTT~~.T..A~~~~~~AA.GT..T.C.....C..
P. caerulea                     TCCTG.A.C...T.TTTTT~C.TTTTTTT~~.T..A~~~~~~AA.GT..T.C.....C..
P. menispermifolia              TCCTG.A.C...T.TTTTT~C.TTTTTTT~~.T..A~~~~~~AA.GT..T.C.....C..
P. oerstedii                    TCCTG.A.C...T.TTTTT~C.TTTTTTT~~.T..A~~~~~~AA.GT..T.C.....C..
P. lobata                       TCTTGGA.C...T.TTTTT~~~~~~~~~~~..T..A~~~~~~AA..T..T.C.....C..
P. mollissima                   TCCTG.A.C...T.TTTTT~C.TTTTTTT~~.T..A~~~~~~AA.GT..T.C.....C..
P. pittieri                     TCCTG.A.A...T.TTATTTC.CTTTTT~~..T..AA~~~~~AA..TG.T.C.....C..
P. platyloba                    TCCTG.A.C...T.TTTTTTC.TTTTTTT~~.T..ATAAAGTAA.GTA.T.C.....C..
P. quadrangularis               TTCTG.A.C...T.TTTTT~C.TTTTTTT~.~T..A~~~~~~AA.GT..T.C.....C..
P. suberosa                     TCTTG.A.C...T.TTTTT~C.TTTTTTT~..G..A~~~~~~AA..T..T.C.....C..
P. talamancensis                TCTTG.A.C...T.TTTTT~C.TTTTTT~~..T..A~~~~~~AA..T..T.C.....C..
P. vitifolia                    TCCTG.A.C...T.TTTTT~C.TTTTTTT~~.T..A~~~~~~AA.GT..T.C.....C..

                                490        500        510        520        530        540
                                ....|....|....|....|....|....|....|....|....|....|....|....|
Sphaerocardamum nesliiforme     TTCGAAGTTTAAGAA~~~~~CTAATATTCATTGATCAAATGATTCACTT~~~~~CATAGT
P. alata                        ...T.....G.....AGGAT.G......A..........C.A.TGA.CAAATAA...AC
P. ambigua                      ...T.....G.....AGGAT.G......A..........C.A.TGA.CCAATAA...AC
P. auriculata                   ...T.....GC....AGGAT.G......A..........C.A.TGA.C~~~~AA...AC
P. biflora                      ...T.....GC....AGGAT.G......A..........C.A.TGA.C~~~~AA...AC
P. coraicea                     ...T.....CC....AGGAT.G......A..........C.A.TGA.C~~~~AA...A.
P. edulis                       ...T.....G.....AGGAT.G......A..........C.A.TGA.CAAATAA...AC
P. caerulea                     ...T.....G.....AGGAT.C......A..........C.A.TGA.CAAATAA...AC
P. menispermifolia              ...T.....G.....AGGAT.G......A..........C.A.TGA.CAAATAA...AC
P. oerstedii                    ...T.....G.....AGGAT.G......A..........C.A.TGA.CAAATAA...AC
P. lobata                       ...T.....GC....AGGAT.G......A~~........C.A.TGA.C~~~~AA...AC
P. mollissima                   ...T.....G.....AGGAT.G......A..........C.A.TGA.CAAATAA...AC
P. pittieri                     ...A.....G.....AGGAT.G......A............A.TGA.C~~~~AA...AC
P. platyloba                    ...T.....G.....AGGAT.G......A..........C.A.TGA.CAAAGAA...AC
P. quadrangularis               ...T.....G.....AGGAT.G......A..........C.A.TGA.CAAATAA...AC
P. suberosa                     ...T.....CC....AGGAT.G......A..........C.A.TGA.C~~~~AA...A.
P. talamancensis                ...T.....GC....AGGAT.G......A..........C.A.TGA.C~~~~AA...AC
P. vitifolia                    ...T.....G.....AGGAT.G......A..........C.A.TGA.CAAATAA...AC
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                                550       560       570       580       590       600
                                ....|....|....|....|....|....|....|....|....|....|....|
Sphaerocardamum nesliiforme     CTGATAGATC~CTTGGTGG~~AACTTATTAATCGGACGAGAATAAAGATAGAGTCCCA~T
P. alata                        G.AC.CC..AGTC..A.A.AT....G.....................................~.
P. ambigua                      G.AC.CC..AGTC..A.A.AT....G.....................................~.
P. auriculata                   G.AC.CC..AGTC..A.A.AT..T.G.....................................~.
P. biflora                      G.AC.TC..AGTC..AGA.AT..T.G.....................................~.
P. coraicea                     G.AC.CC..AGTC..A.A.AT..T.G.....................................~.
P. edulis                       G.AC.CC..AGTC..A.A.AT....G.....................................~.
P. caerulea                     G.AC.CC..AGTC..A.A.AT....G.....................................~.
P. menispermifolia              G.AC.CC..AGTC..A.A.AT....G.....................................~.
P. oerstedii                    G.AC.CC..AGTC..A.A.AT....G.....................................~.
P. lobata                       G~~C.CC..AGTC..A.A.AT..T.G.....................................~.
P. mollissima                   G.AC.CC..AGTC..A.A.AT....G.....................................~.
P. pittieri                     A.AC.CC..AGTC..A.A.AT....G...C.................................~.
P. platyloba                    G.AC.CC..AGTC..A.A.AT....G.....................................~.
P. quadrangularis               G.AC.CC..AGTC..A.A.AT....G.....................................~.
P. suberosa                     G.AC.CC..AGTC..A.A.AT..T.G.....................................~.
P. talamancensis                G.AC.TC..AGTC..AGA.AT..T.G.....................................NA.
P. vitifolia                    G.AC.CC..AGTC..A.A.AT....G.....................................~.

                                610       620       630       640       650       660
                                ....|....|....|....|....|....|....|....|....|....|....|....|
Sphaerocardamum nesliiforme     TTTACATGTCAATACTGACAACAATGAAATTTATAGTAAGATG
P. alata                        .C............TC........G.................G.AAAATCCGTCGACTTNA
P. ambigua                      .C............TC.....T..G.................G.AAAATCCGTCGACTTTA
P. auriculata                   .C............TC........G........G.......G.AAAATCCGTCGACTTTT
P. biflora                      .C............TC........G........G.......G.AAAATCCGTCGACTTTA
P. coraicea                     .C............TC........G........G.......G.AAAATCCGTCGACTTTA
P. edulis                       .C............TC........G.................G.AAAATCCGTCGACTTTA
P. caerulea                     .C............TC........G.................G.AAAATCCGTCGACTTTA
P. menispermifolia              .C............TC........G.................G.AAAATCCGTCGACTTTA
P. oerstedii                    .C............TC........G.................G.AAAATCCGTCGACTTTA
P. lobata                       .C............TC........G........G.......G.AAAATCCGTCGACTTTT
P. mollissima                   .C............TC........G.........N......G.AAAATCCCGTCGACTTT
P. pittieri                     .C............TC........G........G.......G.AAAATCCGTCGACTTTA
P. platyloba                    .C............TC.....T..G..............A.G.AAAATCCGTCGACTTTA
P. quadrangularis               .C............TC........G.................G.AAAATCCCGTCGACTTT
P. suberosa                     .C............TC.
P. talamancensis                .C............TC.G......G........G.......G.AAAATCCGTCGACTTTA
P. vitifolia                    .C............TC........G.................G.AAAATCCGTCGCTTTAG

                                670       680       690
                                ....|....|....|....|....|....|....|
Sphaerocardamum nesliiforme
P. alata                        GAAATCGTGAGGGTTCAAGTCCCTCTATTCCCCAA
P. ambigua                      GAAATCGTGAGG
P. auriculata                   GAAATCGTGA~~
P. biflora                      GAAATCGGGGGGTTCAAGTCCCTTTTTCCCCAAA
P. coraicea                     GAAATCGTGAGGGTTCA
P. edulis                       GAAATCGTGAGGGG
P. caerulea                     GAAATCGTGAGG
P. menispermifolia              GAAATCGTGAGGGTTCA
P. oerstedii                    GNNNTCNTNNGGG
P. lobata                       GAAATCTGAGGTCA
P. mollissima                   AGAAATCGTGAG
P. pittieri                     GAAATCG
P. platyloba                    GAAATCGTGAGGGTTCAAGTC
P. quadrangularis               ANAAATCGTG
P. suberosa
P. talamancensis                GAAATCGTGAGGGT
P. vitifolia                    AAAT
```

APPENDIX G

ACCESSION NUMBERS

Passifloraceae

tRNA-Leucine Intron

AF454778	*P. alata*
AF454779	*P. ambigua*
AF454780	*P. auriculata*
AF454781	*P. biflora*
AF454782	*P. coriacea*
AF454783	*P. edulis*
AF454784	*P. caerulea*
AF454785	*P. menispermifolia*
AF454786	*P. oerstedii*
AF454787	*P. lobata*
AF454788	*P. mollissima*
AF454789	*P. pittieri*
AF454790	*P. platyloba*
AF454791	*P. quadrangularis*
AF454792	*P. suberosa*
AF454793	*P. talamancensis*
AF454794	*P. vitifolia*
AF461415	*P. tica*

ITS1/5.8S/ITS2 Region

AF454795	*P. menispermifolia*
AF454796	*P. vitifolia*
AF454797	*P. oerstedii*
AF454798	*P. platyloba*
AF454799	*P. quadrangularis*
AF454800	*P. alata*
AF454801	*P. ambigua*
AF454802	*P. caerulea*
AF454803	*P. edulis*
AF454804	*P. auriculata*
AF454805	*P. biflora*
AF454806	*P. suberosa*
AF454807	*P. coriacea*
AF454808	*P. lobata*
AF454809	*P. talamancensis*

Heliconiinae

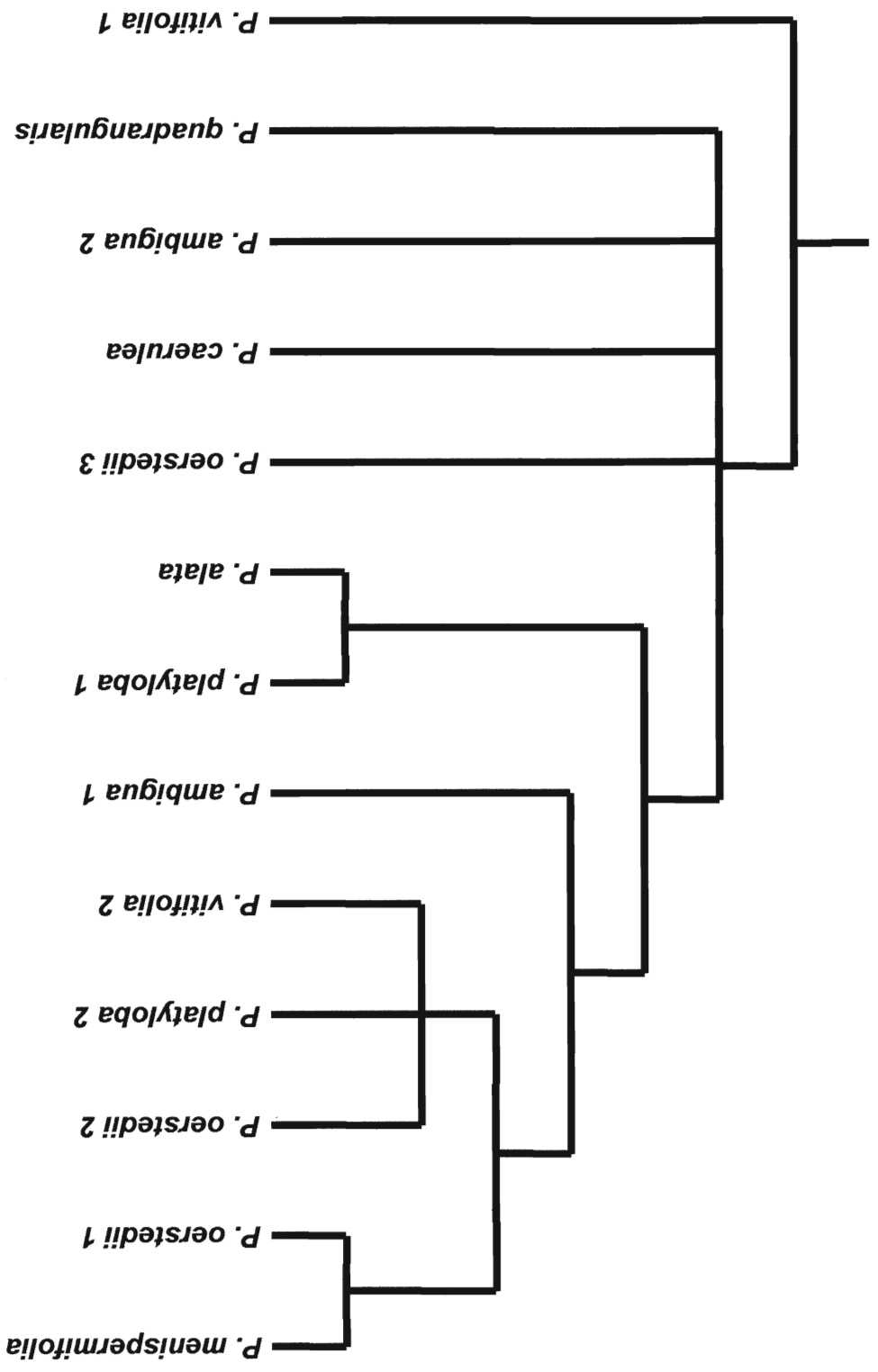
Partial EF-1 alpha

AF454810	*Agraulis vanillae*
AF454811	*Dryadula phaetusa*
AF454812	*Dryas iulia*
AF454813	*H. charithonia*
AF454814	*L. doris*
AF454815	*H. eleuchia*
AF454816	*H. erato*
AF454817	*H. hecale*
AF454818	*H. ismenius*
AF454819	*H. melpomene*
AF454820	*H. sapho*
AF454821	*H. cydno*
AF454822	*H. hortense*
AF454823	*H. sara*

Insect ITS 2

AF453762	*Agraulis vanillae*
AF453763	*Dryadula phaetusa*
AF453764	*Dryas iulia*
AF453765	*H. erato*
AF453766	*H. cydno*
AF453767	*H. melpomene*
AF453768	*H. hecale*
AF453769	*H. ismenius*
AF453770	*H. eleuchia*
AF453771	*H. sara*
AF453772	*H. sapho*
AF453773	*H. charithonia*
AF453774	*H. hortense*
AF453775	*L. doris*

APPENDIX H



Alternate topology of Host-Usage Cladogram analysis for *Passiflora* (*Granadilla*) + *Distephana* Feeding Group.

APPENDIX I

Glossary of Terms

Autapomorphy	A character state form which is found in only one terminal taxon.
Bootstrap Value	The percentage of times each branch is present in the most parsimonious topologies from the re-sampled data sets.
Bremer Decay Index (BDI)	Bremer decay indices are the number of extra steps required from the most parsimonious topology to find an alternative topology where a particular branch is not present (Bremer 1988).
Clade	A monophyletic group including a node and all nodes descendant from it.
Cladogenesis	The evolutionary splitting of lineages (i.e. speciation).
Consistency Index (CI)	A measure of how well character data matrices fit tree topology (testing for homoplasy (i.e. how many times a character evolves on a tree)). The maximum CI value of one is reached if there is no homoplasy.
Ingroup	The focal group of taxa that are assumed to share ancestral species (outgroup(s)).
Monophyletic Group	A phylogenetic lineage which consists of two or more taxa including the common ancestral taxon and all descendants. Also called a clade.
Node	The point at which a lineage branches or ends.
Outgroup	One or more taxa that are assumed to be phylogenetically outside or ancestral to the ingroup.
Phylogenetic tree	A diagrammatic representation portraying the hypothesized evolutionary relationships and sequences of events occurring between taxa.
Retention Index (RI)	A measure of how well character data matrices fit tree topology which accounts for characters not contributing to the tree topology (e.g. autapomorphies)
Sister group	The taxon that is hypothesized to be most closely related to another taxon.
Treelength	The total amount of change or evolutionary events required to explain the data in a particular tree. Also referred to as "steps".